REMARKS

Applicants submit this Preliminary Amendment in order to respond to the Office Action dated October 21, 2003. Accompanying this Preliminary Amendment is a Request for Continued Examination.

Applicants previously submitted this response along with a Petition to Revive, but the Petition was dismiss on January 12, 2005, on the grounds that the reply did not place the application in condition for allowance. Specifically, in an Advisory Action the Examiner stated that the amendment to claim 12 is not supported in the specification as originally filed. Support for claim 12 is discussed in detail in this preliminary amendment. Applicants respectfully request that if the rejection is maintained the Examiner indicate what aspect of the support is at issue.

In the Office Action, the Examiner noted that the specification should be amended to correct the reference to Figures 2A-2B, 3A-3B, etc. This has been addressed by amendment herein. Applicants have also submitted corrected drawings. Withdrawal of this objection is respectfully requested.

Claim 16 was objected to for being of improper dependent form, and this has been addressed by amendment herein, as has the objection to claim 18 in view of a typographical error.

Claims 15-18 were rejected under 35 U.S.C. § 101 because the claimed invention allegedly is directed to non-statutory matter. The Examiner suggested amending the claims to recite "an isolated polypeptide, comprising at least 14 contiguous amino acids . . . ." Claim 15 has been amended accordingly. The Examiner also stated that the recitation of "epitope bearing portion" was not necessary in view of the recognition in the art that a protein of 5-6 amino acids is sufficient to generate an antibody. Claim 15 has been amended accordingly.

Claims 15-18 allegedly fail to include limitations that would distinguish the claimed proteins from those found in nature. The Examiner suggested amending the claims to recite a purity limitation.

Applicants have amended claims 14 and 15 as suggested by the Examiner, as supported in the specification at, for example, at page 13, lines 6-9. However, for completeness of the record, applicants have addressed the case law cited by the

Examiner. *Diamond v. Chakrabarty,* 206 USPQ 193 (1980), did establish the "hand of man" as being relevant in the patentability of genetically engineered microorganisms. *Ex parte Siddiqui,* 156 USPQ 246 (1966), was cited in support for the statement that purity of a naturally occurring product does not necessarily impart patentability. *Siddiqui* relates to a compound obtained by a series of "extractions and neutralizations." In contrast, applicants did not obtain FGF-23 simply by extracting it from a natural source. Instead, as described in the specification, it is a product of a laboratory construct not found in nature, namely, DNA obtained by amplification of cDNA, cloned into a vector, and expressed as a recombinant protein in insect cells. The Examiner has offered no evidence that FGF-23 is obtainable through a method analogous to that described in *Ex Parte Siddiqui.* However, *Merck Co. v. Chase Chemical Co.,* 273 F. Supp. 68 (1967), was cited to support the statement that when purity results in a new utility, patentability is considered. By analogy, applicants have provided isolated FGF-23 that is novel. Reconsideration and withdrawal of this rejection are respectfully requested.

Claims 12-18, 22 and 61-65 were rejected under 35 U.S.C. § 101 because the claimed invention allegedly has no specific and substantial credible utility. Responding to the arguments and Declaration filed on June 3, 2003, the Examiner stated that the specification does not disclose use of the claimed invention for lowering serum phosphate levels, nor which diseases the claimed invention could be used for treating. Applicants request reconsideration and withdrawal of the rejection.

At page 7, lines 28-29, the specification specifically cites, and incorporates by reference, *Nature Genetics* 26:354-358 (2000), which discusses disorders of phosphate metabolism. According to Dr. Kavanaugh's Declaration, of record, administration of a non-cleavable FGF-23, as supported in the specification, lowered serum phosphate levels in mice. This finding is consistent with studies described in the *Nature Genetics* publication. The publication describes families afflicted with the disease known as autosomal dominant hypophosphataemic rickets, in which patients exhibit low serum phosphorous concentration, among other symptoms. The authors describe a mutation analysis designed to identify linkages between carriers of the disease, and mutations in specific genes. They found missense mutations in a member of the fibroblast growth factor family of proteins, which they identify as FGF23. The authors further report this to

be the first mutation identified in the FGF family (page 347, last full paragraph). However, they did not identify the mechanism of action.

Thus, it is of record in the application, by virtue of the *Nature Genetics* article incorporated by reference, that a mutation in FGF-23 is associated with a disease state that manifests as lower serum phosphate. The present applicants have identified a mechanism by which this occurs, by replicating the decreased serum phosphate level phenotype in mice administered with FGF-23 engineered to contain an amino acid change that prevented cleavage of the FGF-23. This is discussed in detail in the Declaration of Dr. Kavanaugh, of record and as filed on June 3, 2003.

In the Office Action, the Examiner states at page 6, paragraph 11, that the asserted utility was not substantial, because further research allegedly was needed to confirm a utility. The Examiner also stated that no specific benefit exists in currently available form.

Applicants respectfully disagree. A disease, autosomal dominant hypophosphataemic rickets, discussed above, is linked to mutations in FGF-23 at precisely the location that applicants have identified as a physiologically important cleavage point. Subsequent publications have further confirmed that FGF-23 plays a substantial role in regulating phosphate levels in the human and other mammals. See, for example, Ward, L.G., *et al., Bone* 34:905-911 (2004) (hypophosphatemic rickets resolved following removal of an FGF-23 producing tumor); Shimada, T. *et al., J. Bone Mineral Res.* 19:429-435 (2004) (FGF-23 is a regulator of phosphate metabolism *in vivo*, and FGF-23 injection reduced serum phosphate levels); Blumsohn, A., Curr. *Opin. Nephrol. Hypertens.* 13:397-401 (2004) (FGF-23 knock-out mouse shows hyperphosphatemia); and Shimada, T. *et al., Endocrinology* 143:3179-3182 (2002) (confirming the present applicants' results showing that mutant FGF-23 was not cleaved between positions 179 and 180, and that mutant FGF-23 expressed by cells implanted in mice caused hypophosphatemia and decrease in bone mineral content). These publications are filed herewith as Exhibits 1-4.

Finally, although clinical trials are not required in order to confirm the utility of an invention, applicants note that a clinical trial by the NIH (NIDCR) is underway to study the role of FGF-23 in phosphorous regulation (Exhibit 5). Such clinical trials would not

take place absent *in vitro* and animal studies providing abundant evidence of a role for FGF-23 in this precise pathway.

For the foregoing reasons, reconsideration and withdrawal of the rejection under 35 U.S.C. § 101 are respectfully requested.

Claims 12-13 and 61-65 were rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite in view of the language "wherein said polypeptide retains the biological activity of human FGF-23." In support, the Examiner cited several cases, two of which date from 1948-49. *Ex parte Wu,* 100 USPQ 2d 2031, 2033 (Bd. Pat. App. & Inter., 1989), was cited for use of the term "such as." The Examiner stated that the following aspect of this case is relevant here: is the language merely exemplary and not required, or is it a required feature of the claims? The *Wu* decision actually construes the term "optionally," not the term "such as," which was interpreted in a prior case that the *Wu* Board distinguished in reaching a decision that Wu's claims were in fact not indefinite under 35 U.S.C. § 112, second paragraph. Furthermore, the Board in *Wu* stated that the determination of compliance with §112, second paragraph, "necessarily depends on the facts of each particular case or application," (100 USPQ 2d at 2033). Since claims 12-13 and 61-65 as amended herein contain neither "such as" or "optionally" or similar terms, *Wu* is inapplicable. The Examiner also cited three other cases, none of which support the ground of rejection. *Ex Parte Steigewald,* 131 USPQ 74 (Bd. App. 1961) does not support the Examiner's position because the present claims do not recite the term "such as," and as pointed out in reference to *Wu*, the facts of each particular case are important in interpreting a phrase for compliance with §112, second paragraph. *Ex parte Hall,* 83 USPQ 38 (Bd. App. 1948). also fails to support the position because it construes the term "such as," which is not recited in the present claims. Finally, *Ex parte Hasche,* 86 USPQ 481 (Bd. App. 1949), is not on point because it construes the terms "which may be" and "such, for example, as" which are not recited in the present claims.

Reconsideration and withdrawal of this ground of rejection are respectfully requested.

Claim 13 was rejected under 35 U.S.C. § 102(b) as being anticipated by Smallwood *et al.*, (*P.N.A.S.* 93:9850-9857, 1996). In view of the amendment to claim 13 herein, applicants submit that this ground of rejection may be withdrawn.

All of the claims remaining in the application are now believed to be allowable. Favorable consideration and a Notice of Allowance are earnestly solicited.

If questions remain regarding this application, the Examiner is invited to contact the undersigned at (206) 628-7650.

Respectfully submitted,
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Bone 34 (2004) 905–911

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Resolution of severe, adolescent-onset hypophosphatemic rickets following resection of an FGF-23-producing tumour of the distal ulna☆

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Received 15 September 2003; revised 16 December 2003; accepted 22 December 2003

Abstract

Oncogenic hypophosphatemic osteomalacia (OHO) is an uncommon hypophosphatemic syndrome characterized by bone pain, proximal muscle weakness and rickets. It has been postulated that OHO results from overproduction of a humoral phosphaturic factor by an occult tumour. Recently, some OHO tumours have been shown to elaborate fibroblast growth factor-23 (FGF-23), which causes renal phosphate wasting when administered to mice. The purpose of this study was to undertake detailed investigations to confirm the diagnosis of OHO in a pediatric patient and to document the biochemical, radiographic and bone histological phenotype before and after tumour removal. We describe an 11-year-old, previously healthy girl with significant pain and functional disability associated with hypophosphatemic rickets. Circulating 1,25-$(OH)_2$ vitamin D was very low (14 pM; *N*: 40–140) while the FGF-23 serum level was markedly elevated [359.5 reference units (RU)/ml, *N*: 33–105]. An iliac bone biopsy revealed severe osteomalacia, but periosteocytic lesions, as are typical for X-linked hypophosphatemic rickets, were not seen. Sequence analyses of the *PHEX* and *FGF23* genes were normal. A radiographic skeletal survey revealed a small exostosis of the left, distal ulnar metaphysis. A tumour was subsequently removed from this site and the pathology was consistent with benign, fibro-osseous tissue. Serum FGF-23 was normal when measured at 7 h post-operatively, while serum phosphate reached the low-normal range at 16 days following surgery. An iliac bone biopsy taken 5 months after the operation showed improvement, but not yet resolution, of the osteomalacia. Biochemical parameters of bone and mineral metabolism suggested that complete resolution of the osteomalacia was not achieved until 12 months following surgery. One year after tumour removal, the patient was pain-free and had resumed a normal level of activity. The rapid normalization of FGF-23 levels following removal of a benign tumour and the subsequent improvement in the biochemical and histological parameters of bone and mineral metabolism suggest that FGF-23 played a key role in this girl's disease.

Keywords: Tumour-induced osteomalacia; FGF-23; Children; Bone histology; Rickets

Introduction

Oncogenic hypophosphatemic osteomalacia (OHO) is an acquired, paraneoplastic syndrome that results in markedly deranged mineral and skeletal metabolism. The disorder is characterized by hypophosphatemia due to renal phosphate wasting, osteomalacia, bone pain, proximal muscle weakness, fractures and functional disability. A very low circulating 1,25-$(OH)_2$ vitamin D level despite hypophosphatemia is the biochemical hallmark of the disease [4]. OHO is commonly associated with small, slow-growing tumours of mesenchymal origin that may be difficult to detect [4]. These tumours are thought to produce a circulating phosphaturic factor [6]. If the causative tumour can be located and completely removed, there is normalization of serum phosphate and remission of the bone disease. OHO is an uncommon entity in children, with fewer than 20 pediatric cases reported in the literature to date [4,21].

OHO shares similarity with two genetic diseases, X-linked hypophosphatemia (XLH) and autosomal domi-

☆ This study was supported by the Shriners of North America.

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doi:10.1016/j.bone.2003.12.025

nant hypophosphatemic rickets (ADHR), and may be difficult to distinguish from the genetic hypophosphatemias on clinical grounds. XLH results from mutations in the *PHEX* (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) gene [11], which encodes a membrane-bound endopeptidase, whereas ADHR is associated with mutations in the *FGF23* gene, which encodes a phosphaturic factor by the same name [1].

Here we report the case of an 11-year-old girl with severe OHO due to an inconspicuous, fibro-osseous neoplasm and describe the clinical, biochemical and bone histological course of the disease before, and up to 12 months following, excision of the tumour. We further provide evidence for the role of fibroblast growth factor-23 (FGF-23), a circulating phosphaturic factor [18,19], in the pathogenesis of this patient's disease.

Clinical report

The patient, a girl of French-Canadian/Scottish descent, first came to medical attention at 11 years of age, when she complained of bilateral knee pain that had gradually developed over a 2-year period. Previously she had been a healthy, elite soccer player with a high level of physical activity. An antalgic gait was noted but no specific diagnosis was made.

By 11.5 years of age, she was wheelchair bound due to significant pain in the hips and knees. At this time, her height was at the 50th percentile, and she was in mid-puberty (Tanner stage III overall). Radiographs of the wrists and knees showed signs of rickets. A Tc99m MDP bone scan revealed numerous foci of increased uptake (mid-humeral shafts, right femoral neck, proximal and mid-right femoral shaft, left tibia, ribs and left sacroiliac joint), which correlated with Looser's Zones on plain radiographs. An extensive evaluation for suspected malignancy was negative. However, low levels were found for serum inorganic phosphorus (0.6 mM; *N*: 1.0–1.7 mM), the threshold maximum for renal tubular phosphate reabsorption/glomerular filtration rate (TmP/GFR, 0.25 mM; *N*: 0.78–1.94) and serum 1,25-$(OH)_2$ vitamin D (14 pM; *N*: 40–140), whereas serum levels of ionized calcium, intact PTH and 25-OH vitamin D were normal. Serum alkaline phosphatase was elevated (631 U/l; *N*: 105–420). There was no biochemical evidence of a generalized tubulopathy.

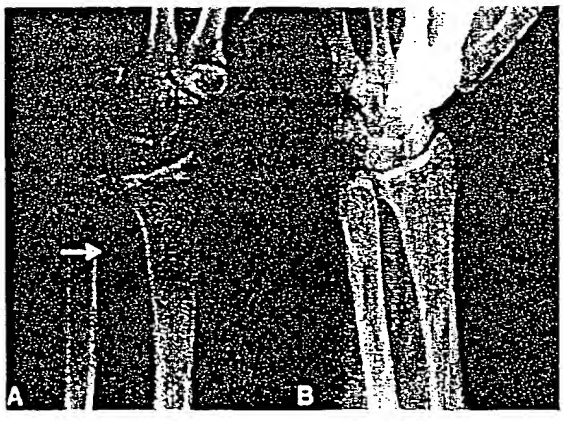

Fig. 1. Radiographs showing (A) subphyseal changes and an exostosis of the left, distal ulna, with an overall "washed-out" appearance of the bone, immediately pre-operatively and (B) fusion of the growth plates with correction of the mineralization defect and the appearance of improved bone density on a plain radiograph taken 12 months following surgery.

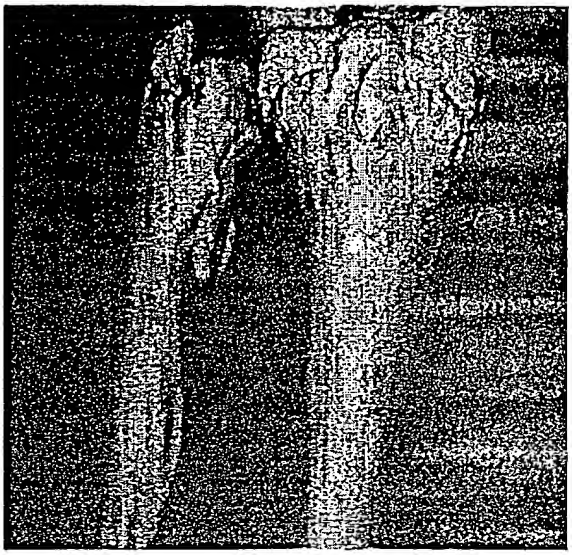

Fig. 2. Three-dimensional, reconstructed computed tomography at 14 years of age showing the exostosis of the left, distal ulnar metaphysis.

A diagnosis of hypophosphatemic rickets was made and treatment was initiated with calcitriol (Rocaltrol, Roche; 0.25 μg twice daily), and sodium acid phosphate (Phosphate-Novartis, Novartis Pharmaceuticals; 500 mg of elemental phosphorus four times daily). Subsequently, her pain improved somewhat and she was able to walk independently, albeit slowly and with a limp.

At 13 years of age, a diagnosis of OHO was suspected, given the ongoing pain and disability despite medical therapy. A radiographic skeletal survey revealed an ill-defined protuberant lesion of the left distal ulnar metaphysis (Fig. 1A), which was confirmed by magnetic resonance imaging and computed tomography (Fig. 2). At the age of 14.8 years, medical therapy was withdrawn and the tumour was removed surgically 1 week later. Detailed investigations were undertaken to confirm the diagnosis of OHO and to chart the changes before and after tumour removal.

Methods

PHEX and FGF23 genetic analyses

Since the biochemical features of OHO resemble those of ADHR and XLH, *PHEX* and *FGF23* mutation analyses were undertaken. For analysis of the *PHEX* gene, genomic DNA was extracted from anticoagulated blood using QUIamp kits (Qiagen). The coding sequences and the flanking sequences of the 22 exons of the *PHEX* gene were amplified using Taq DNA polymerase (Perkin-Elmer-Cetus) and series of specific oligonucleotide primers [10]. The presence of mutations was screened by heteroduplex analysis of PCR products [22], and was confirmed by nucleotide sequence analysis using Big Dye Terminator cycle sequencing and an ABI prism 3100 DNA sequencer.

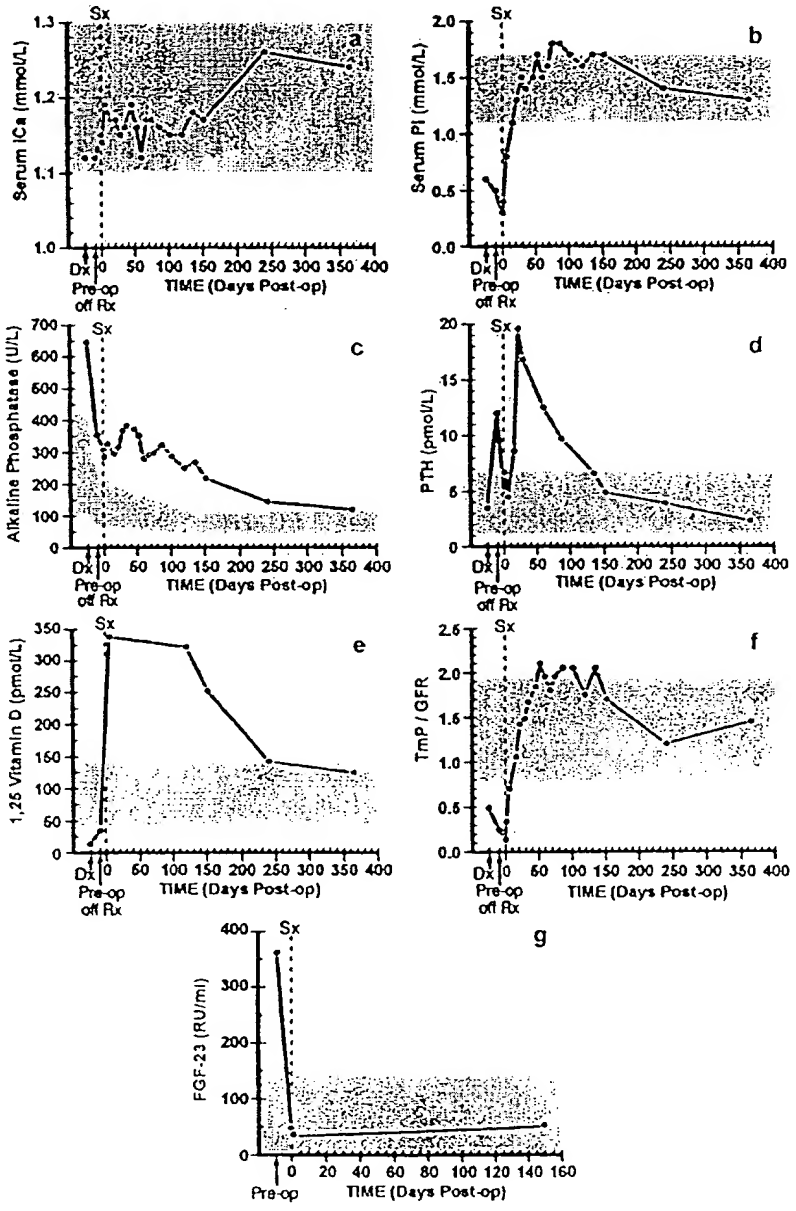

Fig. 3. Serum ionized calcium (a), phosphate (b), alkaline phosphatase (c), intact PTH (d), 1,25-dihydroxyvitamin D (e) and TmP/GFR (f) levels before surgery, and up to 1 year following resection of the FGF-23-producing ulnar tumour. (g) Serum FGF-23 levels pre- and post-operatively, showing rapid normalization of FGF-23 following removal of the tumour.

FGF23 mutation analysis was undertaken in the following manner. The three *FGF23* exons, including the intron–exon splice junctions, were PCR-amplified with intronic primers: (Exon 1 Forward: AATCTCAGCACCAGCCACTC, Reverse: GATGGACAACAAGGGTGCTC; Exon 2 Forward: TTTCAGGAGGTGCTTGAAGG, Reverse: TTGCAAATGGTGACCAACAC; and Exon 3 Forward: CTTCACGTGGTTCGCTCTTG, Reverse: TGCTGAGGGATGGGTTAAAG) using 20 ng of genomic DNA as templates. PCR conditions for all experiments were: 1 min 95°C, followed by 35 cycles of 1 min 95°C, 1 min 57°C, 1 min 72°C, and a final extension of 7 min at 72°C. Amplified exons were analyzed by DNA sequencing with the appropriate forward primers using the ThermoSequenase Kit (USB; Cleveland, OH) and direct incorporation of [^{33}P]dideoxynucleotides. Sequences were resolved on 6% acrylamide gels and autoradiography was performed.

Biochemistry

Serum FGF-23 concentrations were evaluated with a commercially available assay (Human FGF-23 C-Terminal Elisa Kit, Immutopics, San Clemente, CA) according to the manufacturer's instructions. This is a two-site sandwich ELISA that recognizes the C-terminal portion of FGF-23. Results were compared to published pediatric reference data [12]. Serum and urine concentrations of calcium, phosphorus and creatinine as well as serum alkaline phosphatase activity were measured using standard methods. Serum intact parathyroid hormone was determined by immunoradiometric assay (N-tact®; Incstar Corp., Stillwater, MN). 25-OH vitamin D and 1,25-$(OH)_2$ vitamin D were measured with radioimmunoassays (25-Hydroxyvitamin D and 1,25-Dihydroxyvitamin D Osteo SP; Incstar Corp.). All samples were obtained after fasting, and all urine studies except those in the immediate post-operative period were obtained from the second void sample in the morning.

Bone densitometry

Lumbar spine (L2-4) densitometry was performed using a Lunar Prodigy device (Lunar Corp., General Electric; Madison, WI). Bone mineral apparent density was determined according to the method proposed by Kroger et al. [13]. Results were transformed to age- and sex-specific Z-scores using published reference data [20].

Histomorphometry

Full-thickness transiliac bone biopsies were obtained on the 5th day after dual tetracycline labeling (Declomycin, Wyeth-Ayerst Canada Inc., Montréal, Canada) and analyzed as described previously [9]. Quantitative histomorphometric results were compared to reference data as established by this laboratory [9].

Results

A lesion measuring 2.0 cm × 1.2 cm × 0.8 cm was removed from the distal ulna. Pathological examination revealed non-specific fibro-osseous tissue, without evidence of malignancy. Tumour tissue was not available for further studies. At 5 months post-operatively, the patient walked

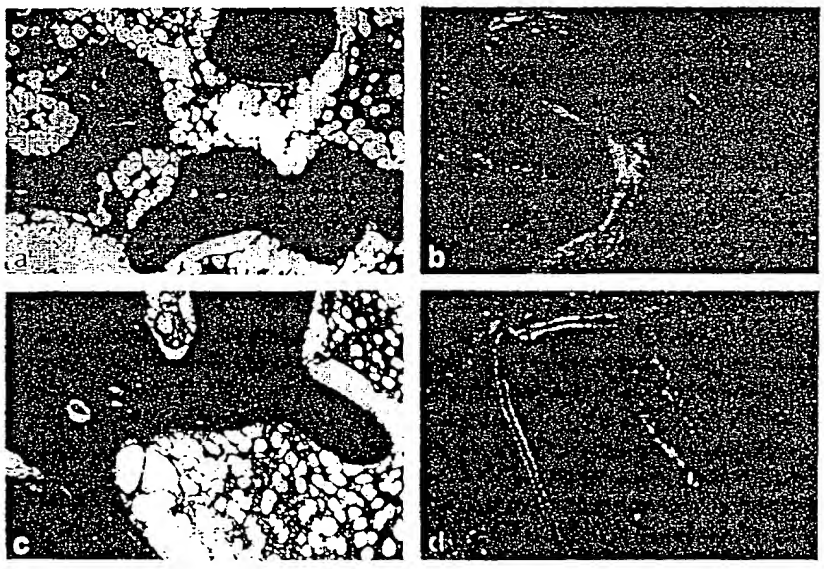

Fig. 4. Transiliac bone histology before surgery, 3 years following initial presentation (a and b), and 5 months post-operatively (c and d). Pre-operatively, there is poor, diffuse uptake of the dual-tetracyline labels due to severe osteomalacia. Peri-osteocytic lesions, characteristic of XLH, were not seen. Five months post-operatively, there is improvement in the osteomalacia with vigorous bone formation.

Table 1
Iliac crest bone histomorphometry before and 5 months after tumour removal

Parameter	Before tumour removal (age 14.0 years)	Percent of age-matched mean* (before tumour removal)	After tumour removal (age 15.2 years)	Percent of age-matched mean* (after tumour removal)
Structural parameters				
Core width (mm)	8.7	122.5	7.4	86.0
Cortical width (μm)	1002.0	111.7	1567.0	133.0
Bone volume/tissue volume (%)	24.7	101.2	29.8	116.0
Trabecular thickness (μm)	172.0	116.2	299.0	190.4
Trabecular number (/mm)	1.4	82.4	1.0	62.5
Formation parameters				
Osteoid thickness (μm)	36.4	543.3	12.7	201.6
Osteoid surface/bone surface (%)	95.8	435.5	62.0	238.5
Osteoid volume/bone volume (%)	42.2	2010.0	5.1	232.0
Mineralizing surface/bone surface (%)	**	**	57.7	461.6
Mineral apposition rate (μm/day)	**	**	0.98	121.0
Bone formation rate/bone surface ($\mu m^3/\mu m^2$/year)	**	**	207.0	559.5
Mineralization lag time (day)	**	**	13.9	90.8
Resorption parameters				
Eroded surface/bone surface (%)	5.2	34.7	20.1	111.7
Osteoclast surface/bone surface (%)	0.2	22.2	0.8	82.7

*Mean values according to Ref. [9].
**Unable to calculate due to severe osteomalacia, resulting in poor uptake of tetracycline label.

with a normal gait and had resumed low-level physical activity. She was pain-free by 12 months after the operation and was able to engage in her usual sport activities.

Sequence analyses of the *PHEX* and *FGF23* genes revealed no mutations. Peri-operative biochemical analyses are presented in Figs. 3a–f. Serum phosphate rose to the lower limit of the reference range by 16 days following surgery. The alkaline phosphatase initially rose following tumour removal, before undergoing a gradual decline. Normalization of the alkaline phosphatase was not achieved until 12 months following surgery. Intact PTH rose to a peak at 3 weeks following surgery, and normalized by 5 months after tumour excision. At 24 h post-operatively, serum 1,25-$(OH)_2$ vitamin D rebounded to 9-fold above the immediate pre-operative value, and declined to the upper limit of normal by 12 months after surgery. Serum FGF-23 was significantly elevated before tumour excision [359.5 reference units (RU)/ml; *N*: 33–103], with normalization when measured at 7 h post-surgery (Fig. 3g). FGF-23 remained within the normal range upon re-evaluation 5 months later.

Bone mineral apparent density at L2-4 increased from 0.266 g/cm^3 pre-operatively to 0.350 g/cm^3 at 5 months after the operation, corresponding to an increase in Z-score from −2.5 to −0.8. Twelve months following surgery, there was fusion of the growth plates with correction of the mineralization defect (Fig. 1B).

An iliac bone specimen obtained 8 months before tumour excision showed severe osteomalacia, with a large amount of osteoid and poor uptake of dual-tetracycline labels. There was no evidence of periosteocytic lesions, the histological hallmark of XLH. Five months following excision of the tumour, osteoid indices had markedly decreased and there was elevated bone formation activity (Fig. 4 and Table 1).

Discussion

We describe an adolescent girl with severe OHO due to a small, histologically benign tumour that secreted high levels of FGF-23. OHO may be difficult to distinguish from XLH and ADHR on clinical and biochemical grounds. However, the disease should be suspected in the pediatric patient with hypophosphatemic rickets when there is significant pain and weakness, a negative family history of hypophosphatemia, and a very low 1,25-$(OH)_2$ vitamin D level. Although our patient's normal height and straight limbs were also suggestive of an acquired process, short stature and skeletal deformity are not universal in ADHR and XLH [7,8]. Both ADHR and XLH may not manifest until the late- or post-pubertal years, and may be associated with a mild phenotype [7,8,16]. In this situation, evaluation of the *FGF23* and *PHEX* genes may provide the diagnostic clue. OHO can be further distinguished from XLH on the basis of bone histological findings, as periosteocytic lesions are commonly present in XLH, but not in other forms of osteomalacia [14]. These periosteocytic lesions are halos of unmineralized bone surrounding osteocyte lacunae and are thought to indicate a primary osteoblast defect in XLH [5,14].

Serum FGF-23 is elevated in many patients with OHO, but also in XLH [12], and thus is of limited use for the

differential diagnosis. In OHO, however, FGF-23 levels can be expected to normalize as early as 30 min following removal of the offending tumour [23], allowing for rapid verification of complete surgical excision. Serial FGF-23 measurements may prove useful in the future, for long-term monitoring of disease recurrence. Indeed tumour recurrence has been reported up to 17 years after initial removal of an OHO lesion [3]. In our patient, while FGF-23 was rapidly cleared following tumour removal, serum phosphate did not normalize until 16 days after surgery. The reason for the relatively slow normalization of serum phosphate following tumour removal is unclear.

A low serum 1,25-$(OH)_2$ vitamin D level is the biochemical signature of OHO, a phenomenon which occurs despite hypophosphatemia [4]. Extracts of OHO tumours have been shown to inhibit the production of 1,25-$(OH)_2$ vitamin D [2], through suppression of renal 1-alpha-hydoxylase activity [15], which may be a direct effect of FGF-23 [17]. The significant rise in 1,25-$(OH)_2$ vitamin D following tumour removal and normalization of FGF-23 are consistent with this hypothesis. However, it remains unclear why OHO patients do not manifest hypocalcemia in the presence of depressed 1,25-$(OH)_2$ vitamin D levels. Our patient's precipitous rise in PTH post-operatively corresponded with the time at which the serum phosphate had reached the lower limit of normal. This observation is consistent with the hypothesis that transient hyperparathyroidism following OHO tumour excision results from disinhibition of PTH secretion following resolution of the hypophosphatemia.

Biochemical parameters of bone metabolism correlated with our patient's clinical symptomatology, as she reported resumption of a normal lifestyle once the alkaline phosphatase level approached the upper limit of normal. Mineralization of osteoid was directly documented by a decrease in osteoid indices and indirectly by the rapid increase in bone mineral apparent density. This was accompanied by normalization of the mineralization lag time and vigorous bone formation activity. The potential for restitution of mineralized skeletal tissue following excision of an OHO tumour during youth is reflected in the 31% increase in spinal bone mineral apparent density that occurred during the 5-month post-operative period.

In summary, we describe a teenage girl with severe OHO who experienced cure following excision of a benign, fibro-osseous lesion. This case highlights the importance of considering OHO in the differential diagnosis of children and adolescents who present with hypophosphatemic syndromes. While serum phosphate normalized by 16 days following tumour removal, biochemical and histological parameters of bone and mineral metabolism suggested that complete reversal of the mineralization defect was not achieved until 1 year post-operatively. The rapid normalization of FGF-23 following removal of the OHO tumour suggests that FGF-23 played a key role in the pathogenesis of this patient's disease.

Acknowledgments

We thank the following individuals at the Shriners Hospital for Children in Montréal, Québec: Guy Charette and Josée Dépot for assistance with bone biopsy sample processing, and Mark Lepik and Guylaine Bédard for the figures and photography.

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JOURNAL OF BONE AND MINERAL RESEARCH
Volume 19, Number 3, 2004
Published online on December 29, 2003; doi: 10.1359/JBMR.0301264

FGF-23 Is a Potent Regulator of Vitamin D Metabolism and Phosphate Homeostasis

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ABSTRACT: We analyzed the effects of an FGF-23 injection in vivo. FGF-23 caused a reduction in serum 1,25-dihydroxyvitamin D by altering the expressions of key enzymes for the vitamin D metabolism followed by hypophosphatemia. This study indicates that FGF-23 is a potent regulator of the vitamin D and phosphate metabolism.

Introduction: The pathophysiological contribution of FGF-23 in hypophosphatemic diseases was supported by animal studies in which the long-term administration of recombinant fibroblast growth factor-23 reproduced hypophosphatemic rickets with a low serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] level. However, there is no clear understanding of how FGF-23 causes these changes.
Materials and Methods: To elucidate the molecular mechanisms of the FGF-23 function, we investigated the short-term effects of a single administration of recombinant FGF-23 in normal and parathyroidectmized animals.
Results: An injection of recombinant FGF-23 caused a reduction in serum phosphate and 1,25$(OH)_2$D levels. A decrease in serum phosphate was first observed 9 h after the injection and was accompanied with a reduction in renal mRNA and protein levels for the type IIa sodium-phosphate cotransporter (NaPi-2a). There was no increase in the parathyroid hormone (PTH) level throughout the experiment, and hypophosphatemia was reproduced by FGF-23 in parathyroidectomized rats. Before this hypophosphatemic effect, the serum 1,25$(OH)_2$D level had already descended at 3 h and reached the nadir 9 h after the administration. FGF-23 reduced renal mRNA for 25-hydroxyvitamin D-1α-hydroxylase and increased that for 25-hydroxyvitamin D-24-hydroxylase starting at 1 h. In addition, an injection of calcitriol into normal mice increased the serum FGF-23 level within 4 h.
Conclusions: FGF-23 regulates NaPi-2a independently of PTH and the serum 1,25$(OH)_2$D level by controlling renal expressions of key enzymes of the vitamin D metabolism. In conclusion, FGF-23 is a potent regulator of phosphate and vitamin D homeostasis.
J Bone Miner Res 2004;19:429–435. Published online on December 29, 2003; doi: 10.1359/JBMR.0301264

Key words: **FGF-23, phosphatonin, phosphate metabolism, vitamin D metabolism, type IIa sodium-phosphate cotransporter**

INTRODUCTION

TUMOR-INDUCED OSTEOMALACIA (TIO), autosomal dominant hypophosphatemic rickets (ADHR), and X-linked hypophosphatemic rickets (XLH) share common clinical features, such as hypophosphatemia, inappropriately low serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] levels for hypophosphatemia, and rickets/osteomalacia.[1,2] It has been postulated that an abnormally excessive action of an unidentified humoral phosphaturic factor sometimes referred to as "phosphatonin" may be a common pathogenic mechanism of these disorders.[1,2] Recently, FGF-23 was identified as a causative factor of TIO[3–5] as well as a gene responsible for ADHR.[6–8] In addition, a recent demonstration of a high serum level of FGF-23 in patients with XLH suggested the involvement of FGF-23 in the development of this hypophosphatemic disease as well.[9,10] The pathogenic role of FGF-23 has been shown by the finding that continuous administration of recombinant FGF-23 reproduced hypophosphatemia, inappropriately low 1,25$(OH)_2$D, and rickets/osteomalacia in animals.[5,11] However, it remains unclear how FGF-23 causes hypophosphatemia and an abnormal vitamin D metabolism.

The direct effect of FGF-23 on the phosphate uptake of renal epithelial cells has been reported in several experiments.[5,12,13] Yamashita et al.[13] reported that the rapid change in phosphate transport activity by FGF-23 was mediated by FGF receptor 3c and the p38 MAP kinase cascade. However, the absence of a direct action of FGF-23 on phosphate uptake[5] and conflicting observations concerning

The authors have no conflict of interest.

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the requirement of heparin for FGF-23 effects[12] have also been demonstrated in similar in vitro studies. Therefore, it is still unclear how these in vitro studies reflect the biological effects of FGF-23 in vivo.

In this study, we analyzed biological events induced by a single administration of purified recombinant FGF-23 protein in vivo and investigated the molecular mechanisms induced by FGF-23, resulting in impaired phosphate and vitamin D metabolism.

MATERIALS AND METHODS

Experimental animals

Male BALB/c mice at 5 weeks of age were purchased (SLC) and housed for 1 week before the following experiments. A purified recombinant FGF-23 protein or the same volume of vehicle was administered into the tail vein through a rapid bolus. At 1, 3, 5, 9, 13, and 24 h after administration, a pair of vehicle- and FGF-23–treated groups ($n = 6$ each) were anesthetized using ether to collect blood samples from the heart. They were then killed, and the kidneys were removed. In some mice, urine samples were also collected using metabolic cages (Sugiyamagen) for 12 h after the injection. To examine the effects of FGF-23 on fasting animals, access to food was prohibited for 24 h before some experiments. To evaluate the effect of the parathyroid hormone (PTH), 8-week-old male Sprague-Dawley rats (Charles River) were subjected to a parathyroidectomy (PTX) with an intact thyroid gland or a sham operation while anesthetized with a combination of pentobarbital (35 mg/kg) and ether. Ten days after the operation, the blood samples were collected from the tail artery, and serum calcium and phosphate levels were determined to confirm the effects of PTX. Only animals exhibiting a serum Ca level of less than 8.0 mg/dl and a phosphate level in excess of 11.0 mg/dl were used. Thirteen days after the surgery, a purified recombinant FGF-23 protein or vehicle was administered into the tail vein of the PTX- or sham-operated rats ($n = 6$ each) through a rapid bolus, respectively, and the blood samples were collected from the tail artery 12 h after the injection. All animals were fed standard rodent chow containing 1.1% P and 1.0% Ca (CLEA) and tap water ad libitum. All studies using animals were reviewed and approved by the institutional animal care and use committee at the Pharmaceutical Research Laboratories, KIRIN Brewery Co., Ltd.

Recombinant FGF-23 protein

The purified recombinant FGF-23 was prepared as described previously.[7] Briefly, the Chinese hamster ovary (CHO) cells expressing full-length human FGF-23 were cultured for 3 days, and the conditioned medium was collected. The recombinant human FGF-23 protein was purified from the conditioned medium by a combination of chromatography using SP-sepharose FF (Amersham Bioscience), Ni-NTA Superflow (QIAGEN), and anti FGF-23 monoclonal antibody-conjugated sepharose. The purified protein was resolved in a 20 mM sodium phosphate buffer (pH 6.7) and 0.3 M NaCl and stored at −20°C. The protein concentration was determined by measuring the absorbance at 280 nm with the molar extinction coefficient, $\epsilon = 18{,}610$ $M^{-1}cm^{-1}$. Approximately 0.13 mg of recombinant FGF-23 was recovered from 1 liter of conditioned media.

Measurement of serum parameters

Serum phosphate and calcium concentrations were determined using the P-test and Ca-test, respectively (Wako). The serum PTH level was determined by Mouse Intact PTH RIA Kit (Immutopics). The serum $1,25(OH)_2D$ level was measured with a RIA Kit (Immunodiagnostic Systems).

Preparation of brush border membrane and Western blotting

The brush border membrane (BBM) was prepared by the method previously reported.[14] The pooled renal cortex in the same group was homogenized, and the supernatant was subjected to the precipitation of BBM. The protein concentration of the suspended BBM fraction was determined by Bradford's standard method. To analyze the NaPi-2a content in the BBM fraction, 20 μg of BBM protein was separated by SDS-PAGE and subjected to Western blot analysis using an anti-NaPi-2a polyclonal antibody that was affinity-purified from the rabbit antisera raised by the synthetic peptide corresponding to the C-terminal sequence of NaPi-2a (LALPAHHNATRL). The signals were detected by an ECL system (Amersham Bioscience).

Probes and Northern blotting

The DNA fragments used as probes for all experiments were prepared from murine kidney or bone cDNA by PCR with the following primer pairs: 25-hydroxyvitamin D-1α-hydroxylase (1αOHase); cagacagagacatccgtgtag and ccacatggtccaggttcagtc, 25-hydroxyvitamin D-24-hydroxylase (24OHase); ctcggaacgtcacctcctta and caggctgctgggaatatctc, type I sodium-dependent phosphate cotransporter; gtaaagaaccctgtgtattcc and ctgcctaagaaatccataat, pit-1; ttctgtgcccttatcgtct and gtggccactggagttgatct, and GAPDH; accacagtccatgccatcac and tccaccaccctgttgctgta. Total RNA was isolated from the frozen tissues using an ISOGEN reagent (Nippongene). Twenty micrograms of RNA samples was electrophoresed and transferred to Hybond N^+ (Amersham Bioscience). A radiolabeled probe was prepared using a Megaprime labeling system (Amersham Bioscience). The membrane was hybridized with a ^{32}P-labeled probe in a PerfectHyb reagent (Toyobo) overnight at 65°C. The blot was washed with a solution of 0.1× SSC and 0.5% SDS for 30 minutes at 65°C. The signals were visualized by the BAS system (Fuji).

Measurement of mouse FGF-23 concentration

Various doses of calcitriol (0.2, 2, or 20 ng/head; Wako) or vehicle was administered to 10-week-old male BALB/c mice intraperitoneally. Four hours after the injection, the blood samples were collected from an orbital cavity. The obtained sera were subjected to the measurement of serum phosphate and FGF-23 levels using an ELISA system for human full-length FGF-23,[9] whose antibodies recognize

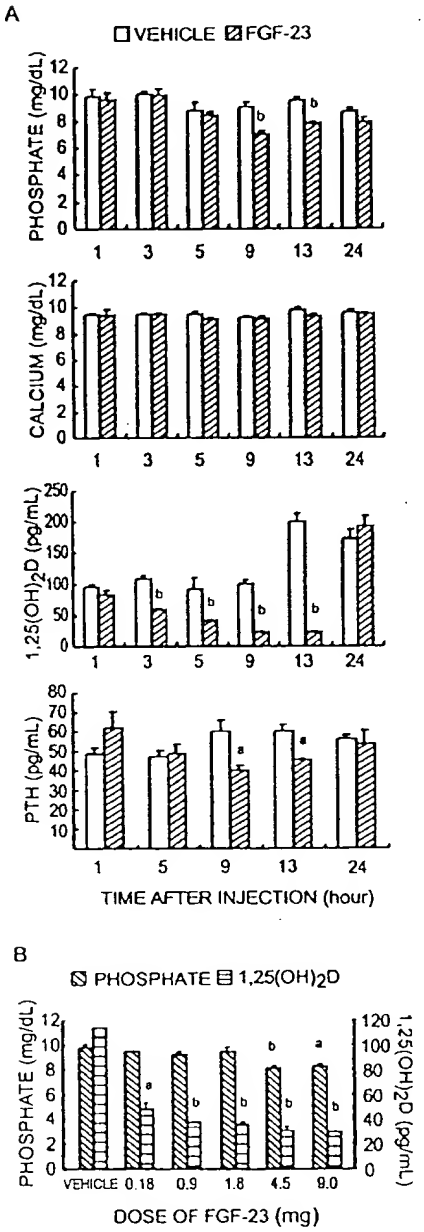

FIG. 1. (A) Time course of changes in serum parameters after treatment with 5 μg of FGF-23. Results are means ± SEM (n = 6). (B) Effects of various doses of FGF-23 on serum phosphate and $1,25(OH)_2D$ levels. Blood samples were collected at 9 h after the injection of an indicated amount of recombinant FGF-23 or vehicle. Results are means ± SEM (n = 6). Statistical analysis was done by (A) Student's t-test and (B) Dunnet's method against the vehicle-treated group. $^{a}p < 0.05$; $^{b}p < 0.01$.

mouse FGF-23 as well as human FGF-23. The measured values were calculated from a standard curve using recombinant human FGF-23 protein.

Statistical analyses

Statistical significance was evaluated either by the Student's t-test or a one-way ANOVA followed by Dannet's method for the comparison of multiple means. An unadjusted p value less than 0.05 was considered to be significant.

RESULTS

Time course of changes in serum parameters

To investigate the time-course of effects of FGF-23 on mineral metabolism, 5 μg of purified human full-length FGF-23 protein was administrated intravenously into normal BALB/c mice, and the blood samples and the kidneys were collected at 1, 5, 9, 13, and 24 h after the bolus injection. As shown in Fig. 1A, the serum phosphate levels of FGF-23–injected mice were significantly lower compared with those of vehicle-treated animals from 9 to 13 h after the injection. The serum phosphate of these groups did not differ at 24 h after the injection. The administration did not affect serum calcium levels at any points examined. Before the changes in serum phosphate level, serum $1,25(OH)_2D$ started to decrease in the FGF-23–injected mice at 3 h after the injection, and this reduction in the $1,25(OH)_2D$ level was maintained for at least 10 more hours. Again, the serum $1,25(OH)_2D$ was not different between the two groups 24 h after the injection. In FGF-23–treated mice, a slight but significant decrease in serum PTH levels occurred at 9 and 13 h after injection, when the serum phosphate levels dropped.

To examine the dose–response relationship of the FGF-23 effects on these changes, serum phosphate and $1,25(OH)_2D$ levels were examined 9 h after the treatment with various amounts of FGF-23 or vehicle. As shown in Fig. 1B, a reduction in serum $1,25(OH)_2D$ was observed in the lowest dose examined (0.18 μg), and serum $1,25(OH)_2D$ decreased in a dose-dependent manner. In contrast, a decrease in the serum phosphate level was observed only at two higher doses (4.5 and 9.0 μg). Thus, FGF-23 could induce a rapid reduction in serum $1,25(OH)_2D$ before the decrease in the serum phosphate levels. However, the diminution of $1,25(OH)_2D$ induced by the lower dose of FGF-23 did not seem to be sufficient for the development of hypophosphatemia.

Effect of FGF-23 on renal phosphate reabsorption

To address the mechanism for the decrease in the serum phosphate level, we first examined the effect of FGF-23 in fasting animals. These animals were not permitted to eat for 24 h to avoid interference of intestinal phosphate absorption. The serum phosphate levels of fasting mice were also significantly reduced compared with those of vehicle-treated animals at 8 h after the injection of 4.5 μg of FGF-23 (vehicle: 7.65 ± 0.15 versus FGF-23: 6.22 ± 0.19 mg/dl, $p < 0.001$, n = 6 each). A reduction in serum $1,25(OH)_2D$ levels by FGF-23 treatment was also observed in the same samples (vehicle: 120.84 versus FGF-23: 28.29 pg/ml, pooled sera, n = 6 each). These findings indicate that the effects of FGF-23 on serum phosphate and $1,25(OH)_2D$ are independent of intestinal phos-

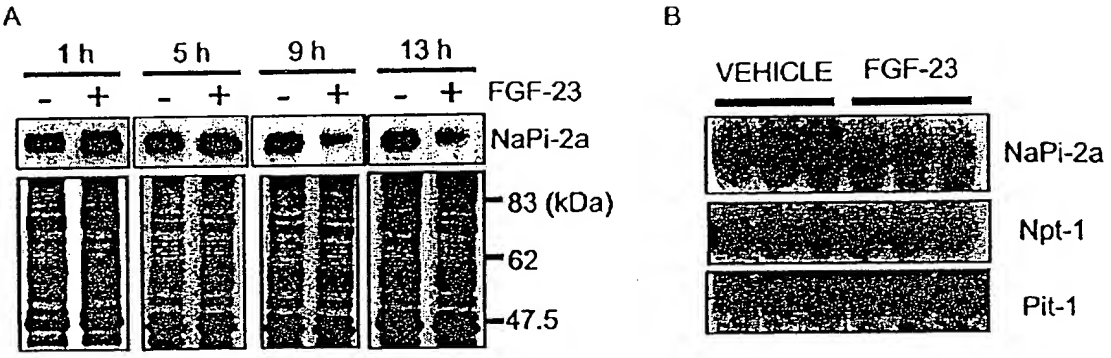

FIG. 2. (A) Western blot analysis for renal NaPi-2a protein. BBM fractions prepared from pooled kidneys in each group at an indicated point were analyzed by Western blot. Immunoreactive bands for anti-NaPi-2a antibody were observed between 60 and 80 kDa (top). The blotted membrane was subjected to Coomassie brilliant blue staining to visualize the amount of blotted protein in each lane (bottom). (B) Northern blot analysis for sodium-dependent phosphate cotransporters. Total RNAs were isolated from the kidney or femur at 9 h after administration of FGF-23.

phate absorption and suggest an important role of the kidney as a target organ of FGF-23 in the regulation of the phosphate and vitamin D metabolism.

It has been demonstrated that the type IIa sodium-phosphate cotransporter (NaPi-2a) in the renal proximal tubules plays a pivotal role in renal phosphate reabsorption.[15,16] Therefore, we next examined the effect of FGF-23 on this molecule. The kidneys obtained from the same groups in the study described above were pooled and used to prepare the BBM fractions. As shown in Fig. 2A, the amount of NaPi-2a protein was clearly diminished at 9 and 13 h after the injection of FGF-23, when serum phosphate also decreased in FGF-23–treated mice. Treatment of FGF-23 also reduced the renal mRNA level of *NaPi-2a* at 9 h (Fig. 2B), whereas neither the mRNA levels of the *type I sodium-dependent phosphate cotransporter* in the kidney nor the *type III sodium-dependent phosphate cotransporter (pit-1)* in the bone changed (Fig. 2B). Consistent with the decreased expression of *NaPi-2a*, the fractional excretion of phosphate for 12 h in mice injected with FGF-23 was higher than that of the vehicle-treated ones (vehicle: 22.6 ± 3.2% versus FGF-23: 34.3 ± 4.9%, $p < 0.05$, $n = 4$ each). Thus, the simultaneous reduction in serum phosphate levels and NaPi-2a amount in the BBM indicate that FGF-23 reduces serum phosphate by inhibiting renal phosphate reabsorption through NaPi-2a.

Regulation of renal vitamin D metabolizing enzymes by FGF-23

To elucidate the molecular mechanism for the reduction in the serum $1,25(OH)_2D$ level, we analyzed the renal expressions of *1αOHase* and *24OHase* genes. Northern blot analysis revealed that FGF-23 decreased the *1αOHase* expression and increased the *24OHase* expression starting as early as 1 h after the administration (Fig. 3A). The quantitative analysis standardized by the expression levels of *GAPDH* indicated that FGF-23 almost halved the *1αOHase* expression and increased the *24OHase* expression by 2.5-fold (Fig. 3B). These alterations lasted more than 9 h after the administration. Because 24OHase is also involved in the degradation of $1,25(OH)_2D$, these rapid changes in mRNA levels in both vitamin D–metabolizing enzymes can be the causative mechanism for the significant reduction in serum $1,25(OH)_2D$ induced by FGF-23.

PTH-independent action of FGF-23

Although PTH is known to be a potent suppressor of NaPi-2a protein expression in the BBM fraction,[17,18] serum PTH levels were not elevated throughout the examined period in this study, as shown in Fig. 1A. These results suggest that the reduction in NaPi-2a protein observed here was not caused by PTH action. To confirm that the biological activity of FGF-23 is independent of PTH, we evaluated the action of FGF-23 in PTX animals. Sprague-Dawley (SD) rats were subjected to parathyroidectomy, and the rats exhibiting significant hypocalcemia and hyperphosphatemia were selected as the PTX ones for the following study (Ca, sham-operated: 10.30 ± 0.18 versus PTX: 6.87 ± 0.17 mg/dl, $p < 0.001$, Pi, sham-operated: 9.03 ± 0.11 versus PTX: 13.42 ± 0.30 mg/dl, $p < 0.001$, $n = 12$ each). In sham-operated rats, recombinant human FGF-23 reduced serum phosphate and $1,25(OH)_2D$ 12 h after the bolus injection of FGF-23 (45 μg/head; Fig. 4). The administration of recombinant FGF-23 to PTX rats significantly improved hyperphosphatemia toward the normal range and further decreased $1,25(OH)_2D$ levels (Fig. 4). These results indicate that the effects of FGF-23 on phosphate and vitamin D metabolism do not require PTH action.

Possible mutual regulatory mechanism between FGF-23 and $1,25(OH)_2D$

This study revealed that FGF-23 can induce rapid and significant reduction in serum $1,25(OH)_2D$ levels, indicating that FGF-23 is a novel hormonal factor playing a critical role in the regulation of the vitamin D metabolism in mammals. It is well known that $1,25(OH)_2D$ has a feedback regulatory mechanism on its own serum level through in-

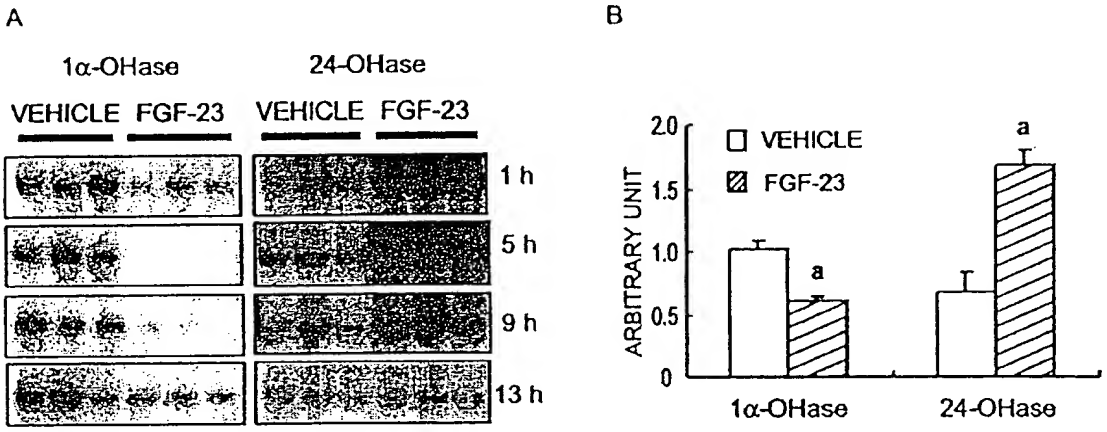

FIG. 3. Effects of FGF-23 on renal expression of vitamin D–metabolizing enzymes. (A) Time course of changes in renal mRNA levels for *1αOHase* and *24OHase* after treatment with FGF-23 or vehicle. (B) Quantitative analysis of the relative abundance of 1αOHase and 24OHase mRNA to GAPDH mRNA at 1 h after injection. Results are means ± SEM. Statistical analysis was done by Student's *t*-test against the vehicle-treated group ($^*p < 0.01$, $n = 5$).

creasing the expression of renal 24OHase and decreasing that of 1αOHase.[19] The results shown above indicate that FGF-23 is deeply involved in the vitamin D metabolism. To investigate the further involvement of FGF-23 in the regulation of the vitamin D metabolism, we examined whether the administration of $1,25(OH)_2D$ could cause changes in serum FGF-23 levels using the ELISA system. This ELISA was originally developed for human FGF-23 and turned out to be applicable to rodent FGF-23.[9] As shown in Fig. 5, a significant elevation of serum FGF-23 levels was observed after 4 h in calcitriol-treated mice. It is noteworthy that even 2 or 20 ng of calcitoriol, which did not change the serum phosphate levels, could evoke the elevation of the serum FGF-23 concentration, consistent with the possibility that the serum FGF-23 level could be regulated by $1,25(OH)_2D$.

DISCUSSION

Previous studies revealed the involvement of FGF-23 in the development of hypophosphatemic rickets/osteomalacia,[3–11] and several reports showed the biological function of FGF-23 in vitro.[5,12,13] However, it has remained unclear how FGF-23 induces these effects in vivo. To address these questions, we investigated the effects of FGF-23 in vivo with time-course experiments.

Concerning the regulation of serum phosphate by FGF-23, we first identified that hypophosphatemia was induced after 9 h of FGF-23 administration. The serum phosphate level is mainly regulated by proximal tubular phosphate reabsorption, and NaPi-2a has been identified as a physiological key molecule that determines renal phosphate reabsorption.[15,16] We have shown here that FGF-23 decreased both serum phosphate and the amount of NaPi-2a protein in the BBM with the same time course. The renal mRNA levels of NaPi-2a also decreased at 9 h. Thus, these results indicate that impaired phosphate reabsorption by FGF-23 is at least in part caused by the reduction in NaPi-2a protein in

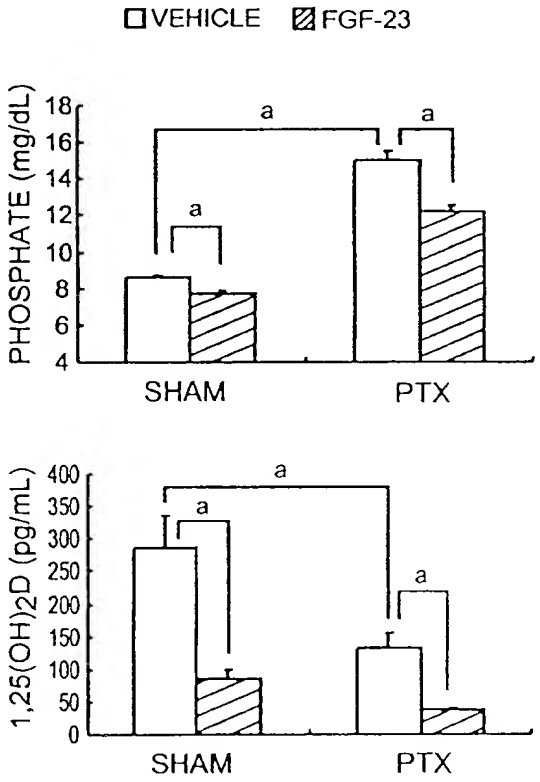

FIG. 4. Effects of FGF-23 in PTX rats. Serum phosphate and $1,25(OH)_2D$ levels at 12 h after injection of FGF-23 or vehicle are shown. Results are means ± SEM. Statistical analysis was done by Dunnet's method. $^*p < 0.01$.

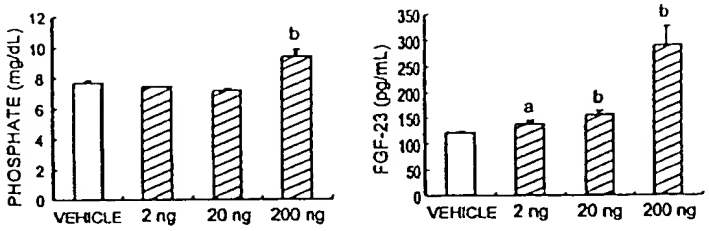

FIG. 5. Effects of calcitriol on serum phosphate and FGF-23 levels. Serum phosphate and FGF-23 levels at 4 h after injection of indicated amounts of calcitriol or vehicle are shown. Results are means ± SEM (n = 4 each). Statistical analysis was done by Dunnet's method against the vehicle-treated group. $^{a}p < 0.05$; $^{b}p < 0.01$.

BBM of renal proximal tubules. Another potent regulator of the decrease in NaPi-2a in BBM is PTH.[17,18] It is possible that PTH might work in the downstream of FGF-23 because PTH can reduce the NaPi-2a protein within 2 h,[18] whereas FGF-23 required more than 5 or 9 h. However, serum PTH levels after the treatment with FGF-23 did not increase, and the FGF-23 action was maintained even in the PTX animals. Therefore, FGF-23 seems to use a PTH-independent pathway to modulate renal phosphate reabsorption. On the other hand, the amount of NaPi-2a protein seemed to be slightly increased at 1 h after the injection of FGF-23 (Fig. 2A). This change was temporary, but it was reproduced in another experiment. It is still unclear whether the rapid increase in NaPi-2a protein is a specific event by the administration of FGF-23 protein. Further studies are required to investigate how FGF-23 modulates the expression of NaPi-2a protein in BBM.

On the other hand, it is possible that FGF-23 also modulates the serum phosphate level by a mechanism that is independent of NaPi-2a. Dietary phosphate loading has been reported to cause the downregulation of NaPi-2a protein.[17] In our preliminary study, a single injection of FGF-23 could decrease the serum phosphate level even in mice fed a high-phosphate diet (1.2% phosphate) for 7 days (vehicle: 11.04 ± 0.55 versus FGF-23: 7.08 ± 0.37 mg/dl, $p < 0.001$, n = 5 each). This result suggests that FGF-23 can regulate phosphate metabolism through an unidentified mechanism that is independent of NaPi-2a. One possibility is that FGF-23 also reduces the recently identified NaPi-2c-dependent renal phosphate reabsorption.[20] Another possibility is that FGF-23 controls extrarenal mechanisms to regulate serum phosphate. Because hypophosphatemic effects by FGF-23 were reproduced in fasting animals, intestinal phosphate absorption is not likely to be the target of FGF-23 action. In addition, FGF-23 injection did not change the mRNA levels for the *type III sodium-dependent phosphate cotransporter, pit-1*, in bone at 9 h. However, it remains unclear whether pit-1 is the primary phosphate transporter in bone, although it has been reported that pit-1 is abundantly expressed in osteoblasts and is suggested to play an important role in the phosphate transport in bone.[21]

Before the reduction in serum phosphate concentration, FGF-23 induced a significant reduction in serum $1,25(OH)_2D$ within 3 h. Such a rapid reduction in the serum $1,25(OH)_2D$ level seems to be caused by a preceding decrease and increase in the expression levels of *1αOHase* and *24OHase* genes, respectively. These changes were maintained even with low serum $1,25(OH)_2D$ and phosphate levels, which, in general, adversely tend to increase *1αOHase* expression and decrease *24OHase* expression. Therefore, one of the primary functions of FGF-23 seems to be to change the expression levels of these enzymes. The presence of such a rapid regulation of serum $1,25(OH)_2D$ levels by FGF-23 was supported by the fact that serum $1,25(OH)_2D$ levels increased soon after the resection of a FGF-23–expressing responsible tumor in a patient with TIO.[9] Furthermore, recently developed FGF-23 KO mice showing high serum $1,25(OH)_2D$ levels show that FGF-23 is an indispensable factor to control serum $1,25(OH)_2D$ levels.[22] It is well known that $1,25(OH)_2D$ has a negative feedback mechanism, in which increased $1,25(OH)_2D$ regulates *1αOHase* and *24OHase* gene expressions directly through a vitamin D receptor.[23,24] On the contrary, our study suggests a negative feedback pathway involving FGF-23 because the administration of $1,25(OH)_2D$ induced the elevation of serum FGF-23 levels. Taken together, these lines of evidence indicate that FGF-23 is a unique and potent downregulator of serum $1,25(OH)_2D$ levels.

It still remains unclear whether a reduction in $1,25(OH)_2D$ by FGF-23 is required for a later decrease in serum phosphate. The reduction in $1,25(OH)_2D$ by FGF-23 occurred in a dose-dependent manner and required less FGF-23 than that required for the induction of hypophosphatemia. Because injections of lower doses, such as 0.18 or 0.9 μg/head of FGF-23 showed a nearly maximum reduction in $1,25(OH)_2D$ but did not affect the serum phosphate levels, the biological activity of FGF-23 needed to reduce serum phosphate levels seems to be different from that needed to decrease serum $1,25(OH)_2D$ levels. One possible explanation is that FGF-23 may have multiple receptor systems with different affinity for the ligand. Alternatively, there may be an unidentified physiological system to maintain the serum phosphate level against the action of FGF-23 to induce hypophosphatemia, and this system is overcome only by treatment with a high dose of FGF-23. To address these issues, further research, such as the identification of the FGF-23 receptor(s) or the establishment of an in vitro assay system(s) reflecting in vivo events, is necessary.

In conclusion, FGF-23 is a unique molecule regulating both phosphate and vitamin D metabolism through novel mechanisms. Further understanding of the biological activity and physiological role of FGF-23 will provide new insights into the homeostatic control of mineral metabolism under normal and abnormal conditions.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and from the Ministry of Health, Labor, and Welfare of Japan.

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Received in original form May 29, 2003; in revised form August 5, 2003; accepted October 10, 2003.

What have we learnt about the regulation of phosphate metabolism?

Aubrey Blumsohn

Purpose of review

The search for hormones which specifically regulate phosphate metabolism has fuelled recent tantalizing studies. These studies have been motivated by diseases involving renal phosphate wasting, including tumor-induced osteomalacia, X-linked hypophosphatemic rickets, and autosomal dominant hypophosphatemia. This review focuses on likely candidate 'phosphatonins' and their possible physiological significance.

Recent findings

Candidate phosphatonins include fibroblast growth factor 23, matrix extracellular phosphoglycoprotein, stanniocalcin, and Frizzled-related protein 4. Fibroblast growth factor 23 has emerged as the prime candidate explaining pathophysiology of these diseases. FGF-23 is expressed in most tumors in tumor-induced osteomalacia. Serum fibroblast growth factor 23 is increased in most patients with X-linked hypophosphatemic rickets and tumor-induced osteomalacia. Injection of recombinant fibroblast growth factor 23 induces phosphaturia, hypophosphatemia, and suppression of 1,25-dihydroxyvitamin D in animals. Many unanswered questions remain, including the relationship between PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) mutations and elevated fibroblast growth factor 23. It is also not clear whether these candidate phosphatonins play a role in phosphate or vitamin D metabolism in healthy humans, or that this role is endocrine. The most compelling evidence derives from the fibroblast growth factor 23-knockout mouse which shows hyperphosphatemia and increased serum 1,25-dihydroxyvitamin D. A physiologically relevant phosphatonin should explain renal adaptation to variable dietary phosphate intake. The tissue source and determinants of serum fibroblast growth factor 23 are unknown.

Summary

Pathophysiological and animal studies serve as a logical foundation on which to base further questions of human physiology. The definition of what is or is not a phosphatonin may need to be refined. There is a need to return to 'old-fashioned' human physiology studies to place recent findings in perspective.

Keywords

adaptation to phosphate intake, fibroblast growth factor 23, PHEX, phosphatonin

Curr Opin Nephrol Hypertens 13:397–401. © 2004 Lippincott Williams & Wilkins.

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Current Opinion in Nephrology and Hypertension 2004, 13:397–401

Abbreviations

ADHR	autosomal dominant hypophosphatemic rickets
FGF-23	fibroblast growth factor 23
FRP4	Frizzled-related protein 4
MEPE	matrix extracellular phosphoglycoprotein
NaPi-2a	type 2 sodium phosphate transporter
$1,25(OH)_2D$	1,25-dihydroxyvitamin D
PHEX	phosphate-regulating gene with homologies to endopeptidases on the X chromosome
PTH	parathyroid hormone
TIO	tumor-induced osteomalacia
XLH	X-linked hypophosphatemic rickets

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Introduction

Although phosphorus is the sixth most abundant element in the body, phosphate metabolism has been slow to yield its mysteries. Serum inorganic phosphate (P_i) is maintained by several mechanisms, including intestinal absorption, interchange with soft tissues and bone, and renal excretion. Although short-term modulation of extracellular P_i can be achieved by transfer between tissue compartments, long-term regulation is dependent on control of renal excretion. About 85% of phosphate filtered at the glomerulus is reabsorbed in the proximal renal tubules, largely by type 2 sodium phosphate transporters (NaPi-2a) [1,2]. The rate of tubular P_i reabsorption depends on the number of membrane NaPi-2a transporters in the proximal tubular brush-border membrane [3•]. Parathyroid hormone (PTH) results in internalization and intracellular degradation of renal NaPi-2a transporters via a mitogen-activated protein kinase pathway [4]. Tubular P_i transport is influenced by several endocrine factors including PTH, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], growth hormone, insulin, thyroid hormone, glucagon, and glucocorticoids. However, these hormones have other primary functions and are only secondarily involved in phosphate metabolism. The search for other endocrine factors ('phosphatonins') which specifically regulate phosphate metabolism has fuelled a series of tantalizing studies over the past few years.

DOI: 10.1097/01.mnh.0000133983.40182.c3

Instructive disease models of renal phosphate wasting

Support for the existence of a phosphatonin hormone derives from rare pathological states involving proximal tubular phosphate wasting, defective skeletal mineralization, and osteomalacia [5–7]. These include tumor-induced osteomalacia (TIO), X-linked hypophosphatemic rickets (XLH), and autosomal dominant hypophosphatemic rickets (ADHR). All are associated with inappropriately reduced serum $1,25(OH)_2D$ despite hypophosphatemia [6]. TIO is a rare paraneoplastic disorder that results in hypophosphatemic osteomalacia, bone pain, fractures, and muscle weakness [8]. The clinical and biochemical features of this disorder resolve following tumor resection. Tumor-conditioned medium contains factors which decrease phosphate transport in renal epithelial cells and reduce $1,25(OH)_2D$ production [5,6,9–11]. The phenotype of XLH and the *hyp* mouse homologue of XLH are also due in large part to a humoral factor [3•]. Renal transplantation does not correct the hypophosphatemic phenotype, and cultured proximal tubular cells from the *hyp* mouse show normal P_i transport. XLH and the *hyp* mouse phenotype are due to inactivating mutations of the neutral endopeptidase PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) [12,13], suggesting that the phenotype could be due to failure to degrade a phosphatonin.

Criteria for definition of a phosphatonin

Kumar [5] proposed a minimal set of criteria for definition of a 'phosphatonin' based on these pathological states. The putative mediator should be shown to (1) inhibit renal tubular phosphate transport, (2) inhibit synthesis of $1,25(OH)_2D$, (3) be produced by tumors in TIO, and (4) be increased in serum in patients with these disorders.

Fulfillment of these criteria does not in itself imply a role in normal phosphate homeostasis, or that this role is endocrine. Susceptibility to degradation by PHEX would also enhance the status of a candidate phosphatonin.

Potential humoral mediators

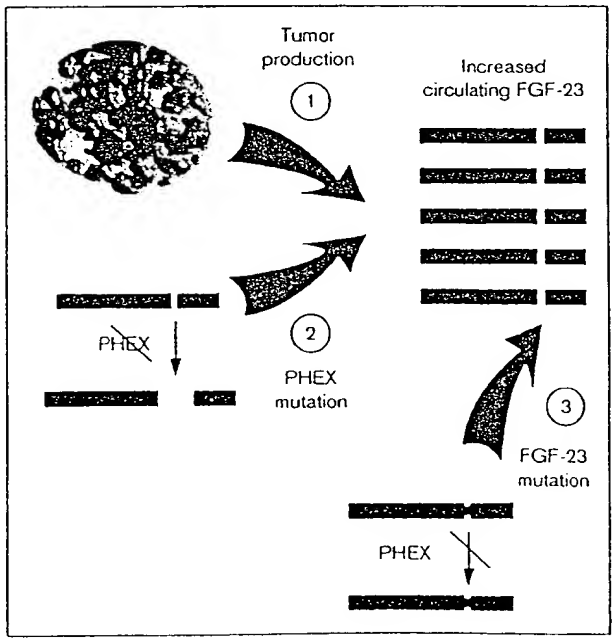
A variety of candidate phosphatonins have emerged over the past few years. These include fibroblast growth factor 23 (FGF-23), matrix extracellular phosphoglycoprotein (MEPE), stanniocalcin, and Frizzled-related protein 4 (FRP4).

Fibroblast growth factor 23

Rapid advances followed from the identification of missense mutations in the gene encoding FGF-23 in ADHR [14]. Mutations are located near a predicted cleavage site suggesting a gain-of-function mutation resulting in impaired FGF-23 degradation [15,16]. Overexpression of an ADHR mutant of FGF-23 in mice leads to greater hypophosphatemia and greater reduction in 1α-hydroxylase activity than seen with overexpression of the wild type [17]. Incorporation of ADHR FGF-23 mutants in rats leads to renal tubular phosphate wasting and suppressed serum $1,25(OH)_2D$ [18].

An initial model [3•,19] explaining the pathogenesis of XLH, ADHR, and TIO with FGF-23 at its core (Fig. 1) was consolidated by several findings. FGF-23 is expressed in most tumors from patients with TIO [20–23]. Serum FGF-23 is increased in XLH [24–26] and TIO [21,24–27], and reduces with tumor resection in TIO [26]. Renal phosphate wasting and reduced expression of NaPi-2a have been demonstrated in transgenic mice expressing FGF-23 [1,28]. Injection of recombinant intact FGF-23 induces phosphaturia and hypophosphatemia in animals [16,29,30•]. Patients with another renal P_i wasting disorder, fibrous dysplasia, also have elevated FGF-23 [31]. FGF-23 has been shown to inhibit renal

Figure 1. Schematic view of proposed mechanisms leading to increased serum fibroblast growth factor 23 (FGF-23) in (1) tumor-induced osteomalacia, (2) X-linked hypophosphatemia, and (3) autosomal dominant hypophosphatemia (ADHR)



In tumor-induced osteomalacia the tumor may secrete excessive amounts of FGF-23. Loss-of-function mutations in PHEX (phosphate-regulating gene with homologies to endopeptidases on the X chromosome) may lead to decreased clearance or perhaps increased synthesis of FGF-23 in X-linked hypophosphatemia. Activating mutations of FGF-23 in ADHR may lead to reduced susceptibility of FGF-23 to proteolytic degradation.

epithelial P_i in some studies [32,33], and to reduce NaPi-2a cotransport activity [18], possibly via a mitogen-activated protein kinase pathway [33]. Injection of FGF-23 in mice also reduces $1,25(OH)_2D$ [30•]. This is in contrast to NaPi-2a gene ablation which results in renal P_i wasting, but a compensatory increase in $1,25(OH)_2D$ [2]. FGF-23-induced suppression of tubular 1α-hydroxylase may involve impaired mRNA translation or modification of enzyme function rather than transcriptional regulation [34].

The ability of PHEX to influence degradation of FGF-23 remains uncertain [32,35••,36,37]. Full-length FGF-23 may not be a PHEX substrate, and it is possible that some form of preprocessing of FGF-23 may be required prior to PHEX-induced cleavage [3,37]. Liu *et al.* [35••] have found that FGF-23 is not a direct PHEX substrate and have presented evidence for an intriguing alternative hypothesis; namely that inactivating PHEX mutations somehow upregulates FGF-23 expression. This would suggest that increased circulating FGF-23 in XLH might be due to increased synthesis rather than reduced clearance. The precise cause of elevated FGF-23 in XLH remains uncertain.

A variety of other mediators apart from FGF-23 may be important. Overexpression of several proteins has been demonstrated in tumors from patients with TIO [38], although some of these may represent downstream effects of other phosphatonin-regulated pathways [3]. Elevated serum FGF-23 has also been found in patients with hypercalcemia of malignancy and primary hyperparathyroidism [39], but the significance of this is uncertain.

Not all patients with XLH or TIO have increased serum FGF-23 [24–27]. This has been taken to suggest that other factors have to be implicated in the pathogenesis of disease in these patients. This is not necessarily the case. 'Normal' serum FGF-23 in some patients could be above a feedback-regulated 'set point' in those patients (just as serum PTH is not elevated in all patients with primary hyperparathyroidism).

Frizzled-related protein 4

FRP4 is expressed by tumors in TIO [38]. Infusion of FRP4 in rats induces hypophosphatemia and may also blunt 1α-hydroxylase and $1,25(OH)_2D$ synthesis [40•]. It seems likely that FRP4 could contribute to the phenotype in TIO, and perhaps XLH.

Matrix extracellular phosphoglycoprotein

MEPE is an extracellular matrix protein which is overexpressed in TIO [38]. Although MEPE fragments do appear to be substrates for PHEX [37], MEPE may not alter P_i transport in renal tubular cells [29]. However, synthetic peptide fragments of MEPE appear to play a role in osteoblast function and bone mineralization and MEPE probably contributes to the skeletal phenotype in XLH and TIO [41].

Stanniocalcin

Stanniocalcin is a calcium-regulating hormone originally thought to be of relevance only in bony fish. Stanniocalcin homologues (STC1 and STC2) are expressed in several mammalian tissues including kidney and bone, and might modulate intestinal absorption and renal transport of phosphate [42–44]. The actions of stanniocalcin in mammals are likely to be paracrine rather than endocrine.

Pathology versus physiology

There is substantial evidence that FGF-23 and possibly FRP4 fulfill the pathophysiological criteria for a humoral 'phosphatonin' proposed by Kumar [5]. It is interesting to speculate that these new molecules are involved in physiological regulation of P_i metabolism in healthy humans, and that this role might be endocrine rather than paracrine. However, despite extensive pathophysiological evidence and animal studies, it is not yet clear that FGF-23, FRP4, or MEPE play a role in the endocrine regulation of P_i metabolism in animals or humans. It is however clear that serum FGF-23 is detectable in most healthy humans [24–26].

It is possible that the status of these phosphatonins in pathology could be analogous to the role of PTH-related protein in hypercalcemia of malignancy. PTH-related protein binds to the PTH receptor and in pathological excess influences serum calcium. However, PTH-related protein has other predominant physiological roles, and its role as a mediator in hypercalcemia of malignancy does not imply an important role in control of serum calcium.

Interestingly, injection of full-length FGF-23 in mice has a suppressive effect on $1,25(OH)_2D$ production that is significantly more rapid and occurs at a substantially lower dose than the effect on tubular P_i handling [30•]. This might suggest that the physiological action of circulating FGF-23 is in the regulation of vitamin D metabolism, and that the effect on P_i metabolism is either pathological or paracrine.

The fibroblast growth factor 23-knockout mouse model

The most compelling evidence that FGF-23 might play a physiological role as a regulator of renal P_i and vitamin D metabolism derives from the FGF-23-knockout mouse model [45••], which shows increased serum P_i, phosphaturia, and increased serum $1,25(OH)_2D$.

Regulatory feedback mechanisms

Although feedback regulation is not a necessary criterion for definition of a hormone, many hormones are controlled by negative feedback systems. The existence of a feedback mechanism influencing the serum concentration of a phosphatonin would lend some credence to the endocrine status of a putative phosphatonin.

A physiologically relevant humoral phosphatonin might explain renal adaptation to variable dietary P_i intake. Adaptation of tubular P_i reabsorption in response to dietary P_i deprivation or dietary P_i loading has been known for several decades [46]. This is due to altered proximal tubular brush-border P_i transport as a result of changes in membrane density of the NaPi-2a cotransporter [47], and is the fundamental backdrop against which an endocrine phosphatonin should be viewed.

If FGF-23 is a physiologically relevant humoral regulator of renal phosphate transport then serum FGF-23 might be expected to alter in response to altered dietary phosphate intake over a relevant timescale, and in a direction consistent with its phosphaturic action. Larsson *et al.* [48] found no effect of 3 days of oral phosphate supplementation (2.4 g/day) on serum FGF-23 in healthy volunteers. However, in another study we found a small but significant increase in serum FGF-23 in men following phosphate supplementation [49].

The tissue source of serum FGF-23, and the way in which P_i might control its production in healthy humans, are unknown. Initial studies found that FGF-23 is expressed at low levels in many tissues including heart, liver, parathyroids, brain, and thymus of adult mice [29,50]. Although some studies have reported no detectable expression of FGF-23 in bone and kidney tissue, expression by human osteoblasts is likely [31]. Liu *et al.* [35••] suggested that serum FGF-23 in XLH might arise from the skeleton due to increased synthesis rather than reduced degradation. The tissue location of tumors in TIO is highly variable, but most are a single histopathologic entity [23] and this may provide some clues as to the likely tissue source of circulating FGF-23 in healthy individuals. If there is a sensing mechanism for dietary phosphorus intake this may not involve serum P_i. One study showed increased serum FGF-23 with phosphorus supplementation even though serum P_i remained stable [49].

It is not clear whether it is necessary to invoke the existence of a novel endocrine factor or 'phosphatonin' to explain other aspects of phosphate metabolism. Circadian changes in renal handling of phosphate are not fully explained by changes in PTH [51], and other mediators may explain this. Although renal glomerular failure is associated with a decrease in phosphate filtration, serum phosphate is initially maintained as a result of a marked decline in tubular phosphate reabsorption. The role of FGF-23 in patients with renal disease is uncertain, and studies are complicated by possible artefactual increases in serum FGF-23 or FGF-23 fragments as a result of renal failure itself. One study showed independent correlations between serum FGF-23 and serum P_i in patients with renal failure [48].

Conclusion

Recent rapid advances in our understanding of the pathophysiology of phosphate wasting disorders have been due to application of a variety of molecular biology techniques and development of transgenic and knockout animal models. Many questions remain to be answered. Is this information of relevance to human phosphate physiology? If candidate phosphatonins are involved in phosphate physiology, do they operate in an endocrine manner? If the phosphatonins are part of a regulatory mechanism, how is this system controlled? What is the predominant tissue source of circulating FGF-23, how is this regulated, and over what timescale do these mechanisms operate? Many of these questions will require a return to 'old-fashioned' human physiology studies in order to place these findings in perspective.

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0013-7227/02/$15.00/0
Printed in U.S.A.

Endocrinology 143(8):3179–3182

Mutant FGF-23 responsible for autosomal dominant hypophosphatemic rickets is resistant to proteolytic cleavage and causes hypophosphatemia in vivo

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FGF-23 is involved in the pathogenesis of two similar hypophosphatemic diseases, autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and tumor-induced osteomalacia (TIO). We have shown that the overproduction of FGF-23 by tumors causes TIO. In contrast, ADHR derives from missense mutations in FGF-23 gene. However, it has been unclear how those mutations affect phosphate metabolism. Therefore, we produced mutant as well as wild-type FGF-23 proteins and examined their biological activity. Western blot analysis using site-specific antibodies showed that wild-type FGF-23 secreted into conditioned media was partially cleaved between Arg^{179} and Ser^{180}. In addition, further processing of the cleaved N-terminal portion was observed. In contrast, mutant FGF-23 proteins found in ADHR were resistant to the cleavage. In order to clarify which molecule has the biological activity to induce hypophosphatemia, we separated full-length protein, the N-terminal and C-terminal fragments of wild-type FGF-23. When the activity of each fraction was examined in vivo, only the full-length FGF-23 decreased serum phosphate. Mutant FGF-23 protein that was resistant to the cleavage also retained the activity to induce hypophosphatemia. The extent of hypophosphatemia induced by the single administration of either wild-type or the mutant full-length FGF-23 protein was similar. In addition, implantation of CHO cells expressing the mutant FGF-23 protein caused hypophosphatemia and the decrease of bone mineral content. We conclude that ADHR is caused by hypophosphatemic action of mutant full-length FGF-23 proteins that are resistant to the cleavage between Arg^{179} and Ser^{180}.

Hypophosphatemia with phosphaturia and inappropriately low serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] are characteristic of three related diseases, X-linked hypophosphatemic rickets/osteomalacia (XLH), autosomal dominant hypophosphatemic rickets/osteomalacia (ADHR) and a paraneoplastic syndrome called tumor-induced osteomalacia (TIO) (1). Because of some evidence that indicates humoral mechanism for the pathogenesis of XLH, a putative circulating phosphaturic factor named 'phosphatonin' has been proposed to be responsible for XLH and TIO (2, 3). On the other hand, the responsible gene for XLH was identified by positional cloning and termed phosphate-regulating gene with homologies to endopeptidases on the X chromosome (PHEX) (4). The protein encoded by this gene is a type II membrane integral protein composed of 749 amino acids. The structural homology and sequence similarity of PHEX to other zinc metallopeptidases such as neprilysin and endothelin-converting enzyme strongly suggest that PHEX has a peptidase activity (5). Therefore, an intriguing hypothesis that PHEX is a critical enzyme to degrade phosphatonin and XLH is caused by excess activity of phosphatonin because of inactivating mutations of PHEX has been proposed (2). However, physiological functions of PHEX protein and the identity of phosphatonin have been unclear.

TIO is a rare paraneoplastic disorder. Once the responsible tumor is identified and removed, abnormal metabolism for both phosphate and vitamin D rapidly disappears. In the previous study, we have cloned FGF-23 as a causative factor of TIO and demonstrated that FGF-23 causes hypophosphatemia, phosphaturia, osteomalacia and decreased 1,25$(OH)_2$D level in vivo (6). FGF-23 was first cloned in mouse as a new member of FGF family by homology to FGF-15 (7). It also has been identified as a responsible gene for ADHR (8). Therefore, FGF-23 is involved in the pathogenesis of at least two related diseases, TIO and ADHR.

In order to investigate molecular mechanisms by which missense mutations found in ADHR cause hypophosphatemia, we prepared wild-type and mutant recombinant FGF-23 proteins. In this study, we demonstrate that wild-type FGF-23 protein is cleaved at a specific site and only full-length FGF-23 has an activity to induce hypophosphatemia. We also show that mutations found in ADHR prevent the cleavage of FGF-23 and a cleavage-resistant mutant FGF-23 protein retains the activity to induce hypophosphatemia.

Abbreviations: ADHR, autosomal dominant hypophosphatemic rickets/osteomalacia; FE, fractional excretion; FGF, fibroblast growth factor; PHEX, phosphate-regulating gene with homologies to endopeptidases on the X chromosome; Pi, inorganic phosphate; TIO, tumor-induced osteomalacia; XLH, X-linked hypophosphatemic rickets/osteomalacia.

Received 2/4/02. Accepted 5/14/02.

Materials and Methods

Cell cultures

A cell line of Chinese hamster ovary cells stably expressing FGF-23 (CHO-FGF23) was established as described (6). CHO-FGF23 cells and PEAKrapid cells (Edge Biosystems, MD) were grown in alpha modified MEM supplemented with 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies, MD). To prepare conditioned media, cells were washed once with PBS and cultured in serum-free SFM-II medium (Life Technologies, MD). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

Expression of recombinant proteins

The cDNA coding FGF-23 with His6 sequence at the C-terminus was prepared as described (6). Expression vectors for mutant FGF-23 were synthesized by in vitro mutagenesis using pEAK8 plasmid (Edge Biosystems, MD). Primers used were as follows; R176Q primers (Forward: 5'-CCCATACCACGGCAGCACACCCGGAG-3', Reverse: 5'-CTCCGGGTGTGCTGCCGTGGTATGGG-3'), R179Q primers (Forward: 5'-CGGCGGCACACCCAGAGCGCCGAGGA-3', Reverse: 5'-TCCTCGGCGCTCTGGGTGTGCCGCCG-3'), R179W primers (Forward: 5'-CGGCGGCACACCTGGAGCGCCGAGG-3', Reverse: 5'-CCTCGGCGCTCCAGGTGTGCCGCCG-3'), R176Q, R179Q primers (Forward: 5'-ATACCACGGCAGCACACCCAGAG-

CGCCGAG-3', Reverse: 5'- CTCGGCGCTCTGGGTGTGCTGCC-GTGGTAT-3'). For transient expression, each plasmid was introduced to PEAKrapid cells using the calcium phosphate method. Cells were cultured for 48 h after transfection and media were harvested. To generate stable cell lines expressing FGF-23 (R176Q, R179Q) mutant protein, pEAK8 plasmid containing the mutant cDNA was introduced into CHO cells and then drug-resistant clones were picked up in the presence of 5 μg/ml puromycin (Sigma, MO).

Separation of recombinant proteins

Conditioned media from CHO-FGF23 cells were filtrated through 0.2 μm membrane (SuporCap, Pall Gelman Laboratory, MI), and then applied to SP-Sepharose FF (Amersham Pharmacia Biotech, Little Chalfont, UK). C-terminal polypeptide fragment of FGF-23 was collected from the flow-through fraction. The retained proteins were eluted with linear gradient of NaCl ranged from 0 to 0.7M. The mature full-length and N-terminal polypeptide fragments were sequentially collected at approximately 0.3 and 0.4 M NaCl. Purified proteins were concentrated into a buffer consisted of 5 mM HEPES and 0.1 M NaCl, pH6.9. Mutant FGF-23 proteins transiently expressed in PEAKrapid cells were purified in the same way.

Antibody generation

Peptides (P-48: RNSYHLQIHKNGHVDGAPHQC and P-148: GMNPPPYSQFLSRRNEC) corresponding to the sequence between Arg^{48} and Gln^{67} and between Gly^{148} and Glu^{163} with additional Cys at the C-terminus were synthesized, conjugated with bovine thyroglobulin and used for immualization of rabbits. Antiserum was collected after 8 times immunization with 2-weeks intervals. Peptides were immobilized on a support (SulfoLink; Pierce, IL) via the side chain of terminal Cys residue and anti-peptide antibodies were affinity-purified using the peptide-coupled gel.

Western blotting

Conditioned media or purified fractions were resolved by 10-20% gradient SDS-PAGE under reduced condition and electroblotted onto a PVDF membrane. The membrane was incubated with anti-His (C-term)-HRP antibody (INVITROGEN, CA) or with polyclonal antibodies described above followed by the incubation with HRP-conjugated anti-rabbit IgG antibody. Signals were detected by ECL system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Analysis of tryptic fragments

Purified FGF-23(R176Q, R179Q) protein was separated on SDS-PAGE gel and stained with Coomassie Brilliant Blue (CBB). Each single band was separately excised from the gel and digested by trypsin (Promega, WI). Molecular mass analyses of tryptic peptides were performed by Matrix-assisted Laser Desorption/Ionization time-of-flight mass spectrometry (MALDI-TOF/MS) using a Voyager-DE/STR (Applied Biosystems, CA). The peptides were identified by comparison of the molecular weight determined by MALDI-TOF/MS and theoretical mass of peptides from FGF-23.

Animals and experimental designs

Purified recombinant protein was intraperitoneally administered to six-weeks old male Balb/c mice (SLC, Japan). To collect urine samples, mice were bred in metabolic cages for 24 hours. Blood samples were taken under anesthesia with diethyl ether. To prepare tumor-bearing mice, approximately 1×10^7 cells were subcutaneously implanted into both sides of backs of six-weeks old male BALB/c athymic nude mice (SLC, Japan). All animals received a commercial rodent diet (CE-2; CLEA, Tokyo, Japan) containing 1.1% phosphate and 1.0% calcium. Diets and tap water were provided *ad libitum* throughout the experiments. All experiments were reviewed and approved by the institutional animal care and use committee at the Pharmaceutical Research Laboratory, KIRIN BREWERY Co., Ltd.

Results

Proteolytic cleavage of recombinant FGF-23

When the recombinant FGF-23 protein with His-tag sequence at the C-terminus was expressed in CHO cells, two recombinant products containing C-terminus were observed and identified as a mature FGF-23 protein lacking signal sequence and a processed polypeptide with Ser^{180} at the N-terminus (6) (Fig. 1A). To further investigate the processing of the recombinant FGF-23 protein, we prepared polyclonal antibodies against the synthetic peptides corresponding to the partial sequence of FGF-23. Both P-48 and P-148 antibodies showed the presence of multiple N-terminal fragments in addition to full-length FGF-23 (Fig. 1A). There seemed to be further processing of N-terminal fragment of FGF-23 besides cleavage between Arg^{179} and Ser^{180}, because P-48 antibody recognized small processed peptides that were not detected by P-148 antibody.

Figure 1. (A) Western blotting analysis of conditioned media from CHO-FGF23 cells using anti His-tag antibody (lane 1), P-48 antibody (lane 2) and P-148 antibody (lane 3). (B) Purified proteins by column chromatography were visualized by the Coomassie Brilliant Blue staining. Lane 1: full-length FGF-23, Lane 2: N-terminal fragments, Lane 3: C-terminal fragment.

Susceptibility of recombinant mutant FGF-23 proteins to the proteolytic cleavage

The preceding amino acid sequence of the cleavage site between Arg^{179} and Ser^{180}, Arg^{176}-His^{177}-Thr^{178}-Arg^{179}, is a consensus sequence of protease recognition motif, RXXR. All three missense mutations of FGF-23 gene in patients with ADHR, R176Q, R179Q and R179W, are in the consensus motif of RXXR (8). To investigate the implication of these mutations in the processing of FGF-23 proteins, several recombinant mutant FGF-23 proteins with substitutions of arginine residues in the RXXR motif were transiently expressed in PEAKrapid cells and analyzed by Western blotting. In contrast to the cleavage of wild-type FGF-23 protein between Arg^{179} and Ser^{180}, all three mutant proteins found in ADHR patients were secreted into conditioned media predominantly as a mature form (Fig. 2A, B). However, the mutant proteins appeared to be heterogeneous on SDS-PAGE. To clarify the cause of this heterogeneity by MALDI-TOF/MS, we generated a mutant FGF-23 protein with double mutations (R176Q, R179Q) that enabled to analyze tryptic fragments around the RXXR sequence by reducing the cleavage sites by trypsin. The recombinant protein with double mutations (R176Q, R179Q) also was resistant to the proteolytic processing between Arg^{179} and Ser^{180} and showed the similar heterogeneity to other mutant FGF-23 proteins (Fig. 2A, B). Four different protein bands around 32.5 kDa (Fig. 2C) were isolated, digested with trypsin and subjected to the mass spectrometric analysis. Results indicated that all four forms retained the full-length polypeptide sequence of FGF-23 (S^{25}-I^{251}) with variations of the number of o-linked sugar chains in the three tryptic fragments (162-175, 176-187, 199-228). All proteins possessed o-linked sugar chain in the 199-228 region. The variation of o-linked glycosylation occurred in the 162-175 region with one

attachment site and the 176-187 region with two attachment sites. The introduction of mutations probably influenced o-linked glycosylation to these attachment sites that are very close to the RXXR motif. These results indicate that mutations found in ADHR result in the resistance to the proteolytic cleavage between Arg^{179} and Ser^{180} by destroying the protease recognition consensus motif, RXXR.

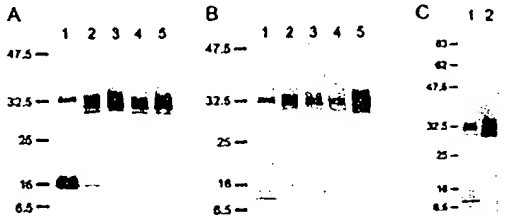

Figure 2. Analysis of mutant FGF-23 proteins by Western blotting. Transient expression of mutant FGF-23 proteins in PEAKrapid cells. Conditioned media were analyzed by Western blotting using P-148 antibody (A) and anti His-tag antibody (B). Lane 1: Wild-type FGF-23, Lane 2: FGF-23 (R176Q), Lane 3: FGF-23 (R179Q), Lane 4: FGF-23 (R179W), Lane 5: FGF-23 (R176Q, R179Q). (C) Conditioned media from a stable cell line expressing wild-type FGF-23 (lane 1) and double-mutant FGF-23 (R176Q, R179Q) (lane 2) were analyzed by Western blotting using anti His-tag antibody.

Biological activity of recombinant FGF-23 products

Since the clinical features of ADHR patients are similar to those of TIO, it is likely that these two diseases derive from a common mechanism of excessive biological activity of FGF-23. To investigate how the processing of the FGF-23 protein influences the biological activity, we first examined the activity of cleaved fragments. Proteins containing full-length mature FGF-23, N-terminal portion and C-terminal fragment of FGF-23 were separated by combination of ion-exchange and metal-affinity chromatography (Fig. 1B). These protein fractions were intraperitoneally administrated into mice twice with an interval of 12 hours. The significant decrease of serum phosphate and increase of renal phosphate excretion (FEPi) were observed only in mice administered mature FGF-23 protein (Table 1). N-terminal and C-terminal fragments of FGF-23 did not affect serum phosphate level or FEPi, either. Serum calcium level did not change by any treatment. These results indicate that the processing between Arg^{179} and Ser^{180} abolishes the activity of the FGF-23 protein to induce phosphaturia and hypophosphatemia.

Table 1. Biological activity of separated recombinant products

	sPi (mg/dl)	sCa (mg/dl)	FEPi
Vehicle	8.10 ± 0.33	9.01 ± 0.08	0.266 ± 0.200
Full length	5.83 ± 0.20**	9.01 ± 0.13	0.390 ± 0.028*
N-fragment	8.91 ± 0.17	9.25 ± 0.08	0.233 ± 0.014
C-fragment	8.94 ± 0.38	8.96 ± 0.13	0.257 ± 0.010

Each protein fraction was administered to mice twice (5 μg each) with an interval of 12 hours (n = 5). At 24 hour after the first administration, fractional excretion of phosphate (FEPi), serum levels of phosphate and calcium were measured. Results are expressed as means +/- SEM. *P<0.005 and **P<0.001 against vehicle by one-way ANOVA followed by Bonferroni's method for comparison of multiple means.

To clarify the relationship between the resistance to the cleavage and biological activity of FGF-23 more directly, we analyzed biological activity of the mutant proteins *in vivo*. Recombinant mutant proteins corresponding to the full-length mature form were purified and administered to mice three times with an interval of 6 hours. Mutant FGF-23 proteins also caused the decrease of serum phosphate level at 24 hours after the first injection to the similar extent to that induced by wild-type FGF-23 (Fig. 3). These results indicated that the mutant protein retained biological activity to induce hypophosphatemia.

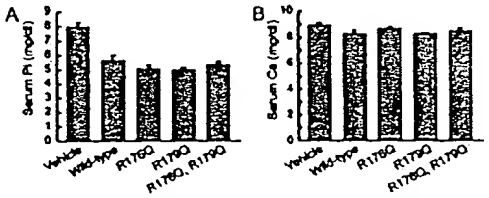

Figure 3. Biological activity of mutant FGF-23 proteins in vivo. Purified full-length mutant FGF-23 proteins (4 μg/infusion) were administered intraperitoneally into mice three times with an interval of 6 hours (n = 4). Blood was obtained at 24 hours after the first injection and serum phosphate (A) and calcium (B) levels were determined. Results are expressed as means +/- SEM.

To examine the long-term effects of mutant FGF-23 protein, we implanted the same number of CHO cells stably expressing the wild-type or mutant protein into athymic nude mice and allowed cells to form tumors. Hypophosphatemia and reduced bone mineral content were observed in both groups (Fig. 4). These results confirmed that the cleavage-resistant FGF-23 protein has an ability to cause hypophosphatemic bone disease.

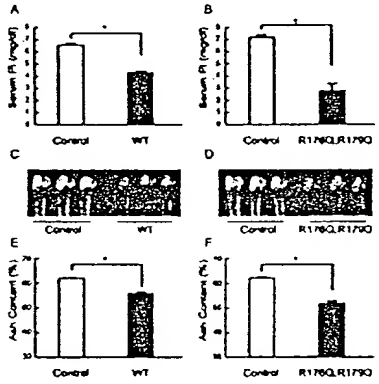

Figure 4. Serum phosphate and bone mineral content of mice with CHO cells stably expressing wild-type or mutant FGF-23(R176Q, R179Q). Wild-type CHO cells were implanted in each experiment as a control. After 44 days, blood samples and femurs were collected. (A) (B) Serum phosphate levels. (C) (D) X-ray images of femurs. (E) (F) The ratio of ash weight to dry weight of femurs (n=3). Results are expressed as means +/- SEM. *P<0.001 by Student's t-test.

Discussion

FGF-23 was identified as a gene responsible for ADHR (8). However, it has been unknown how mutations of FGF-23 cause ADHR. We have shown that overexpression of FGF-23 caused hypophosphatemia and osteomalacia, which are common features to both TIO and ADHR (6). Therefore, we speculated that the mutations of FGF-23 found in ADHR somehow enhance the activity of FGF-23 to induce hypophosphatemia. The proteolytic processing between Arg^{179} and Ser^{180} has been observed when recombinant FGF-23 was expressed in CHO cells (6). Detection of the cleaved C-terminal protein of FGF-23 has also been reported by other groups (9, 10). However, little has been known about the processing of residual N-terminal portions of FGF-23 protein.

In this study, we showed that wild-type FGF-23 is processed at multiple sites especially in N-terminal portion as shown in Fig.1 and most of these proteolytic cleavages occur only after the processing between Arg^{179} and Ser^{180}, because heterogeneity of mutant FGF-23 was derived from differences in the number of sugar chains (Fig. 2). In addition, we demonstrated that neither FGF-23 lacking the unique C-terminal domain nor the C-terminal fragment induced hypophosphatemia (Table 1). These results indicate that the cleavage between Arg^{179} and Ser^{180} is the primary processing of FGF-23 that converts biological active FGF-23 into inactive metabolites in terms of the induction of hypophosphatemia. Furthermore, we demonstrated that all types of mutations found in ADHR patients resulted in the resistance to the processing as described recently (14) and the cleavage-resistant mutations increased the ratio of full-length protein to its inactive metabolites (Fig. 2). We also demonstrated that the cleavage-resistant mutant full-length protein retained the activity to induce hypophosphatemia. These findings suggest that circulatory level of full-length active FGF-23 is increased in patients with ADHR even if expression of FGF-23 is not enhanced.

When the same amount of full-length wild-type and mutant FGF-23 proteins were injected into mice (Fig. 3), wild-type and mutant full-length FGF-23 proteins showed similar potency to induce hypophosphatemia. In contrast, hypophosphatemia and reduced bone mineral content of mice with CHO cells expressing the mutant FGF-23 tended to be more severe than those with wild-type FGF-23 (Fig. 4). This could be explained by higher circulatory level of full-length mutant FGF-23 protein that is resistant to the cleavage. However, it is impossible to directly compare the severity of effects of CHO cells expressing either wild-type or the mutant FGF-23 protein in this model because the expression level of FGF-23 proteins by CHO cell tumors can not be manipulated. Our results indicate that certain amount of either full-length wild-type or the cleavage-resistant mutant FGF-23 causes hypophosphatemic bone disease. Therefore, establishment of more sensitive in vitro assay for biological activities of FGF-23 and measurement of circulatory level of full-length FGF-23 are necessary to compare relative potency and in vivo stability of wild-type and mutant FGF-23 proteins more accurately.

The biological activity of FGF-23 is reminiscent of the putative phosphaturic factor in XLH. The hypothesis that a common phosphaturic factor of TIO and XLH is a substrate for PHEX has been proposed. Although the cleavage of FGF-23 at RXXR motif by PHEX was recently suggested using in vitro translated proteins (10), other sequences than RXXR were suggested as targets of PHEX (11, 12). Recent report also showed that PHEX could not degrade a peptide fragment of FGF-23 containing the RXXR motif (13). In addition, we observed the cleavage of FGF-23 in PEAKrapid cells in which expression of PHEX was not observed by RT-PCR (data not shown). The mutant FGF-23 proteins do not seem to be completely resistant to the cleavage, because small amount of cleaved fragment was still observed (Fig. 2). In addition, the presence of fragments with about 25 kDa was commonly observed as faint signal in lanes with mutant FGF-23 proteins when the exposure period was elongated (data not shown). Additional study is clearly necessary to clarify the relationship between PHEX and FGF-23.

In conclusion, the biological activity of full-length wild-type FGF-23 to induce hypophosphatemia is lost by cleavage between Arg^{179} and Ser^{180}. Mutations in the RXXR motif found in ADHR patients prevent the proteolytic cleavage, and the mutant FGF-23 protein that is resistant to the cleavage retains the activity to induce hypophosphatemia. Therefore, it is concluded that ADHR is caused by hypophosphatemic action of mutant full-length FGF-23 proteins.

Acknowledgments

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This work was supported in part by grants from Ministry of Education, Culture, Sports, Science and Technology, and from Ministry of Health, Labour and Welfare, Japan. We thank Motoko Sato and Ricko Hino for excellent technical support.

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Studying Phosphorus Metabolism

This study is currently recruiting patients.

Sponsored by

National Institute of Dental and Craniofacial Research (NIDCR)

Purpose

Phosphorus and phosphate irons play critical roles in bone structure and essential cellular functions.

The purpose of this study is to learn more about the factors and hormones that regulate phosphorus in the body by collecting blood and urine samples from patients with disorders of phosphate control.

Both children and adults will be enrolled in this study. Researchers will collect blood and urine samples from participants on multiple occasions (2 to 6 times). Some blood specimens will be taken after an overnight fast and participants may be asked to collect all their urine during a 24-hour period. Researchers will analyze these blood and urine samples to better understanding how the body handles phosphorus.

Condition
Phosphorus Metabolism

MedlinePlus consumer health information

Study Type: Observational
Study Design: Natural History

Official Title: Studies in Phosphorus Metabolism

Further Study Details:

Expected Total Enrollment: 100

Study start: August 2, 2003

Phosphorus and phosphate ions play an important role in cellular metabolism as well as bone structure. Scientific evidence suggests that, in addition to Vitamin D and PTH systems, novel factors, such as Fibroblast Growth Factor 23 (FGF-23) and Matrix extracellular phosphoglycoprotein (MEPE), may play an important role in phosphorus regulation. These factors have been best studied in rare genetic and acquired phosphate wasting disorders such

as tumor induced osteomalacia (TIO), X linked hypophosphatemia (XLH) and autosomal dominant hypophosphatemic rickets (ADHR). Patients with other abnormal phosphate regulating states such as hyperparathyroidism and hypoparathyroidism, pseudohypoparathyroidism etc. undergoing phosphorus-altering interventions may provide important insight into the role of these hormones.

We are proposing an observational study with collection of blood and urine samples to study both established (e.g. mineral ions, bone markers, PTH-Vit D system, TMP-GFR) and novel (e.g. FGF-23 and MEPE) constituents of the phosphorus metabolism pathway. Patients with abnormal phosphorus regulating states will be enrolled and we will study the natural history of their disease and the effects of specific interventions that are likely to change phosphorus balance.

The outcome will potentially aid understanding of this new field of mineral regulating hormones and generate both interest and research in phosphorus metabolism. It is hoped that this will also encourage clinical trials in treatment of phosphate wasting disorders.

Eligibility

Genders Eligible for Study: Both

Criteria

INCLUSION CRITERIA:
Patients of any age, gender or ethnicity who will help fulfill the objectives laid out in Section II.
We propose to study patients primarily enrolled in other clinical center protocol(s). They will continue to receive treatment/ interventions per the original protocols.
EXCLUSION CRITERIA:
Patients with significant cognitive impairment who are unable to give informed consent or patients having other significant mineral disturbances that could confound the parameters being studied will be excluded.

Location and Contact Information

Maryland
National Institute of Dental And Craniofacial Research (NIDCR), 9000 Rockville Pike, Bethesda, Maryland, 20892, United States; Recruiting
Patient Recruitment and Public Liaison Office 1-800-411-1222 prpl@mail.cc.nih.gov
TTY 1-866-411-1010

More Information

Detailed Web Page

Publications

Murer H, Forster I, Hernando N, Lambert G, Traebert M, Biber J. Posttranscriptional regulation of the proximal tubule NaPi-II transporter in response to PTH and dietary P(i). Am

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Study ID Numbers: 030254; 03-D-0254
Record last reviewed: February 9, 2004
Last Updated: February 9, 2004
Record first received: August 4, 2003
ClinicalTrials.gov Identifier: NCT00066183
ClinicalTrials.gov processed this record on 2004-10-01
